TAN, XIAO, M.S. Analysis of Ginger Root Essential Oil and Hydrosol: CYP450 Inhibition (2015)
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Former researches showed ginger extracts could contribute to hepatic protection. Two species of cytochrome P450 enzymes, CYP2E1 and CYP2A6, are both important heme-containing liver enzymes for hepatictoxicology via metabolizing small organic molecules into toxic metabolites. CYP2E1 metabolizes more than 2% of all the oral drugs and is closely associated with liver toxicity. CYP2A6 metabolizes fewer than 5% of all the oral drugs and activates some tobacco procarcinogens. Therefore, inhibitors from natural extracts might be able to provide with preventative therapy to liver toxicity and cancer. CYP2E1 inhibitors may be used to inhibit liver toxicity of CYP2E1 in metabolizing the pain killer acetaminophen into toxic N-acetyl-p-quinoimine (NAPQI) to prevent liver cell necrosis. In addition, CYP2A6 inhibitors may be used to suppress the metabolism of certain pro-carcinogens that are inhaled from smoking.

In this research, two extracts from steam distillation of ginger root, ginger essential oil and ginger hydrosol, were collected. CYP2E1 and CYP2A6 were assayed with presence of the two extracts at a series of concentrations to test the ginger extracts' dose-dependent effects. The results showed both of the two extracts as CYP2E1 inhibitors but as poor inhibitors to CYP2A6. The major organic contents of ginger essential oil and ginger hydrosol were identified by GC-MS. The results showed both of

them contained citral. The major organic components of ginger hydrosol were determined as citral (neral and geranial), 2-heptanol and eucalyptol.

The Michaelis-Menten kinetic analysis showed that ginger essential oil, citral and ginger hydrosol were all competitive inhibitors to CYP2E1 from human liver S9 fraction. The K_I for Brazilian ginger essential oil was 27.3mg/L and the K_I for Costa Rican ginger essential oil was 29.9 mg/L. The inhibitory constant of citral was K_I of 43.0μM. Meanwhile, the K_I for Brazilian ginger hydrosol was 4.6% of its original concentration, which corresponded to 45.5 μM citral (neral: geranial=1:1.4).The K_I for the Brazilian ginger hydrosol was 3.6% of its original concentration, which corresponded to 44.3μM citral (neral: geranial=1:1.4). Citral was implicated to be the most potent inhibitor from ginger hydrosol to human liver CYP2E1.

Ginger hydrosol and citral were shown to be inhibitors to purified human liver CYP2E1 in the metabolism of acetaminophen to NAPQI by analysis with LC-MS. In the presence of the substrate acetaminophen at 800μM, the concentration close to its toxic concentration, Brazilian ginger hydrosol exhibited a 50% inhibition of enzyme activity at the concentration over 25%. This concentration of Brazilian ginger hydrosol corresponds to a dose of citral of around 158 μM. This result implicated that ginger hydrosol and citral could be used as a natural therapeutic for toxicity of acetaminophen.

ANALYSIS OF GINGER ROOT ESSENTIAL OIL AND HYDROSOL:

CYP450 INHIBITION

by

Xiao Tan

A Thesis Submitted to
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in Partial Fulfillment
of the Requirements for the Degree
Master of Science

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2015

Approved by

_____
Committee Chair

To my father, Daqing, Tan, and my mother, Meiting, Yao,

I appreciate you forever for your supports. Without your love, I cannot achieve what I have today.

APPROVAL PAGE

This thesis written by Xiao Tan has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Committee Chair_____

Committee Members_____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

I. A. Liver Protection Functions of Ginger Extracts

Ginger (Zingiber officinale) is a flowering plant of which the rhizome was traditionally used as a spicy food and herb for therapy. Extracts from ginger showed anti-inflammation properties via the inhibition of cyclooxygenase-2 (COX-II) [1]. The aqueous extract of ginger rhizome was shown to protect against inducted toxicity of acetaminophen by suppressing the acetaminophen induced reduction of enzymes like glutathione peroxidase or glutathione-S-transferase and thus increase the overall antioxidant levels [2]. Therefore, it is able to inhibit the generation of oxidative stress markers such as lipid peroxidation, which can result in destruction in the plasma membrane and apoptosis. The ginger rhizome extract was reported to inhibit cellular toxicity via enhancing the body's levels of glutathione (GSH), a cellular antioxidant. GSH can conjugate with and neutralize N-acetyl-p-benzoquinone imine, which is formed by metabolism of acetaminophen by certain cytochrome P450 enzyme [2].

I. B. Cytochrome P450 Enzymes

The cytochrome P 450 proteins, i.e. CYP 450, are phase I monooxygenases found in diverse organisms that catalyze mono-oxidation of endogenous and xenobiotic compounds [3]. The cytochrome P450 enzymes were at first named for their significant absorbance at 450 nm in complex with carbon monoxide [4][5][6]. Their active site contains a ferric iron (Fe^{3+}) heme group as shown by Figure 1. 1 in which the central iron coordinates with four nitrogen atoms in the heme plane. A cysteine residue in the bottom axial position attaches the heme to the enzyme via its thiol group's sulfur [7]. In the non-activated form, this Fe^{3+} also coordinates with a water molecule's oxygen at the top axial position as shown by Figure 1. 2.

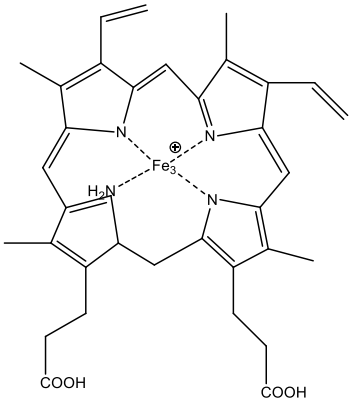

Figure 1. 1. The Iron Heme Group of the Active Site of P450.

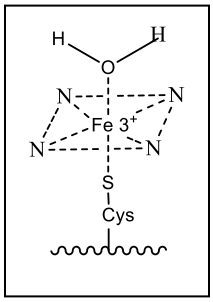

Figure 1. 2. Simplified Heme Group at the Active Site of P450.The ferric iron (Fe^{3+}) is coordinated by four nitrogen atoms in the heme plane and one water molecule's oxygen in the top axial position and one enzyme cysteine residue's thiol sulfur in the bottom axial position. The Fe^{3+} also coordinates with a water molecule's oxygen to the top axial position.

In order to become catalytically active, this heme group needs to be activated by molecular oxygen [8].This whole process was shown by Figure 1. 3. Once activated, this active site heme group is able to catalyze various monooxygenation reactions such as alkane hydroxylation, alkene epoxidization, aromatic epoxidation, dealkylation, etc. Such catalyzed reactions contribute to metabolism of the majority of drugs and zenobiotics and endogenous metabolites [8] [9][10].

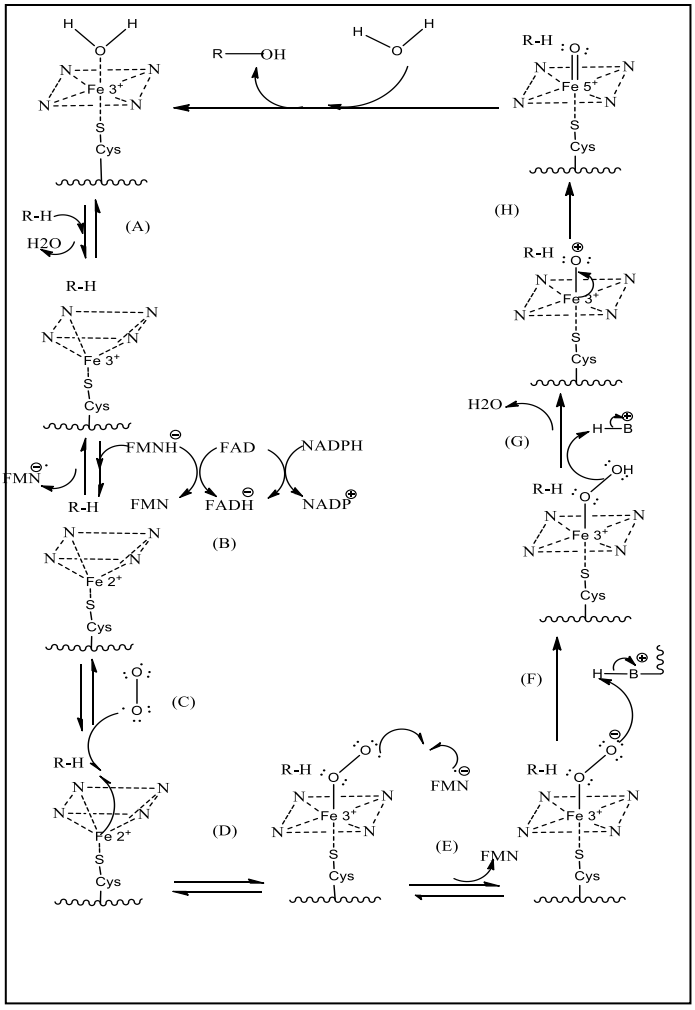

Figure 1. 3. Activation of the Heme Group of CYP450 by Molecular Oxygen. (A): Redox potential enhancement of the ferric iron induced by substrate binding; (B): Nicotinamide coenzyme and flavin coenzyme aided reduction of ferric iron into ferrous iron, i.e. Fe^{2+};(C) (D) (E) (F): Molecular oxygen binding and heme peroxide formation; (G) (H): O-O bound break and formation of highly-oxidative Fe^{5+}oxoferrylheme.

I. C. Cytochrome P450 2E1

Cytochrome P450 2E1 (CYP2E1) is an isoform of the cytochrome P450 super family. Compared to other CYP450 enzymes, CYP2E1 has a relatively small active site which was shown to be around 182 cubic Å [11] as shown in Figure 1. 4. Restricted by the small size of the active sites, the compounds that can be metabolized by CYP2E1are mainly small molecules. These small molecules are mainly metabolized by hydroxylation, which increases their solubility and thus facilitates their excretion [12].Toxic metabolites can be produced by metabolism by CYP2E1 of certain small organic compounds such as toluene [13], halothane, chloroform, pyridine [14], pyrazole [15], acetone, ethanol [16] [17], and acetaminophen [18].Human liver CYP2E1 metabolizes more than 2% of all the oral drug and is closely associated with liver toxicity. Long exposure to CYP2E1 inducers was also implicated to be associated with an increased risk of liver malignancy [19]. The hydroxylation of 4-nitrophenol (PNP) into 4-nitrocatechol shown in Figure 1. 5 was used to assay the activity of CYP2E1. Prior researches implicated that when the total concentration of CYP450 in one reaction was lower than 50μM, the major 4-nitrophenol hydroxylase was CYP2E1. The reaction of hydroxylation of 4-nitrophenol would thus be eligible to be used to assess the activity of CYP2E1.

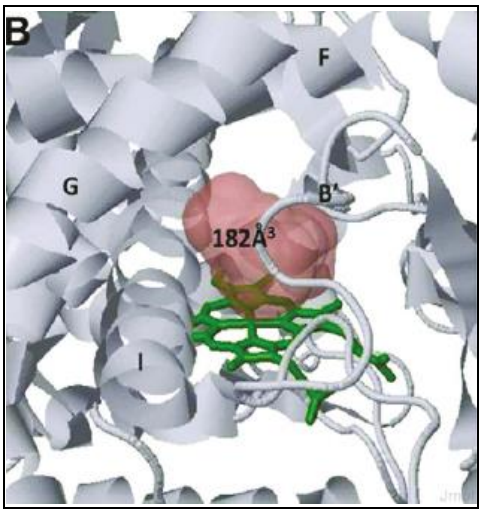

Figure 1. 4. Active Site of Human CYP2E1. The volumes were calculated by online tool Q-Site Finder in the research of Li et al based on Protein Data Bank Code 3e4e [11].

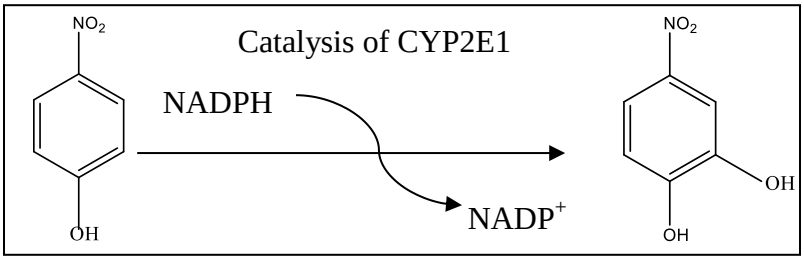

Figure 1. 5. Hydroxylation of 4-Nitrophenol (PNP) into 4-Nitrocatechol Catalyzed by CYP2E1.

I. D. Cytochrome P450 2A6

CYP2A6 was shown to metabolize fewer than 5% of all drugs and catalyzes the activation of certain procarcinogens [20]. Cytochrome P450 2A6 (CYP2A6) metabolizes some tobacco carcinogens and its activity has been associated with development of lung cancer [21]. The hydroxylation of coumarin into 7-hydroxycoumarin shown in Figure 1.6 was recorded as an exclusive reaction catalyzed by CYP2A6 [22] and thus was used to assay the activity of CYP2A6.

Figure 1. 6. Hydroxylation of Coumarin in 7-Hydroxycoumarin Catalyzed by CYP2A6.

I. E. Ginger Essential Oil and Hydrosol

In order to obtain extracts containing relatively small compounds as inhibitors for CYP2E1 and CYP2A6, extracts from distillation were selected as candidates. Such extracts include ginger essential oil and ginger hydrosol. By definition, essential oil is the oil collected from parts of the plant species by distillation. Hydrosol is the fragrant water formed as a byproduct from the steam distillation in essential oil production. Its name comes from a combination where “hydro” means water and “sol” means solution [23]. By property, it is a water solution where some water soluble microdrops of the essential oil and some water soluble volatile components of the plant coexist. The essential oil and hydrosol are separate by their differences in gravity. Therefore, in the distillation operation the essential oil would always be collected from the upper layer of the collector vessel because it is heavier than hydrosol which needs to be collected from the lower layer. [23] Both the essential oil and hydrosol of ginger possess soothing, anti-inflammatory or antiseptic properties [23][24].

I. F. Importance of Searching for CYP2E1 and CYP2A6 Inhibitors from Ginger Extracts

The aqueous extract of ginger rhizome was shown to protect against inducted toxicity of acetaminophen by suppressing the acetaminophen induced reduction of enzymes like glutathione peroxidase or glutathione-S-transferase. In other words, it increased the overall antioxidant levels [2], preventing the occurrence of oxidative stress markers such as lipid peroxidation or destruction of the plasma membrane, which leads to apoptosis. Meanwhile, the ginger rhizome extract was also reported to inhibit cellular toxicity via enhancing the body's levels of glutathione (GSH), a cellular antioxidant that binds to one apoptosis-inducing metabolite of acetaminophen [2].

As for the physiological benefit from ginger extract via restriction of toxicity, although prior studies showed the aqueous extract of ginger rhizome could protect against toxicity of acetaminophen by suppressing the acetaminophen induced reduction of antioxidant enzymes [2], there are still few reports on whether ginger extracts could directly inhibit CYP 2E1 catalyzed generation of liver-toxic metabolism, such as metabolism of acetaminophen into N-aceto-p-benzoquinoimine (NAPQI).

Meanwhile, since the size of CYP2A6 active site is relatively small as shown by Figure 1. 7 [25], we looked for possible CYP2A6 inhibitors from the distillation extracts made from ginger rhizomes. (The molecular sizes of most of the components from the

ginger distillation extracts are small.) Searching for the possible CYP2A6 inhibitor from ginger extracts from steam distillation could help evaluate such extracts' potency as a natural therapeutic in preventing malignancy resulting from smoking.

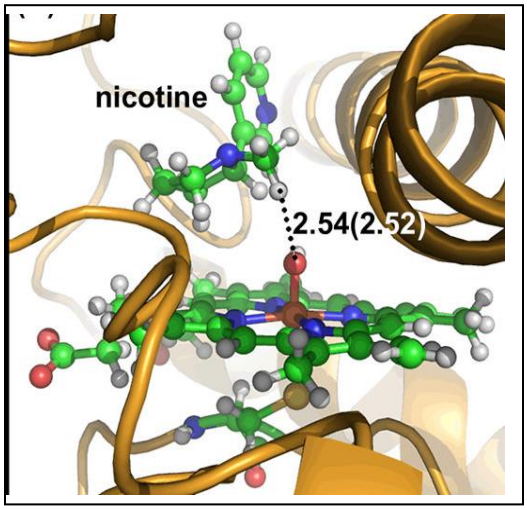

Figure 1. 7. Active Site of CYP2A6 Showing the Heme in Complex with Nicotine. The dimensions were shown in Å.

I. G. Research Method Outline

This research started from making steam distillation extracts from ginger. Two types of extracts were obtained from distillation: ginger essential oil and ginger hydrosol. Dose dependent effects of these extracts were carried out to CYP2E1 and CYP2A6 using the protocols described in prior studies to test whether and how much they inhibited these two cytochrome P450 enzymes [26]. Then the component analysis of ginger essential oil was directly done with GC-MS; the component analysis of ginger hydrosol was done

with a method combining HPLC with GC-MS. New dose response tests of human liver CYP2E1 were done with certain pure compounds that constituted the components of ginger essential oil and hydrosol. Further tests were performed to assess the potential of ginger hydrosol in inhibiting the metabolism of acetaminophen into N-aceto-p-benzoquinoimine (NAPQI) by human liver CYP2E1 (Figure 1. 8). Since NAPQI is an unstable compound, its quantification could not be performed before it was converted to a relatively stable complex. This purpose was achieved by conjugating NAPQI with reduced glutathione (GSH) into NAPQI-GSH conjugate. This reaction was catalyzed by glutathione-S-transferase (provided by human liver cytosol) via conjugating NAPQI with GSH (Figure 1. 9). The GSH was added at high concentration in order to convert all of the NAPQI formed into conjugate in order to evaluate the CYP2E1 activity accurately [22].

This research would finally show whether compounds from ginger could be possible protector of the liver by direct inhibition of metabolic generation of toxic metabolites. The further research of the candidate CYP2E1 inhibitory chemicals could also assess their effect in protection of liver cells against toxicity from acetaminophen metabolism.

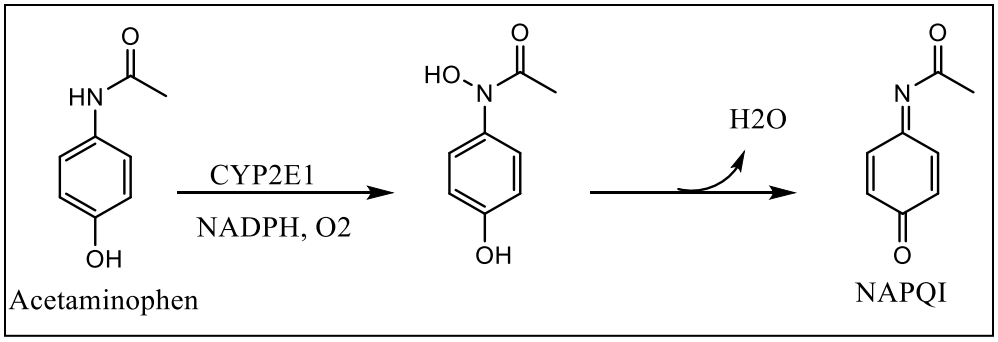

Figure 1. 8. Metabolism of Acetaminophen into NAPQI Catalyzed by CYP2E1. Acetaminophen is at first be hydroxylated by CYP2E1. Then this hydroxylated intermediate formed would lose one molecule of water to form NAPQI.

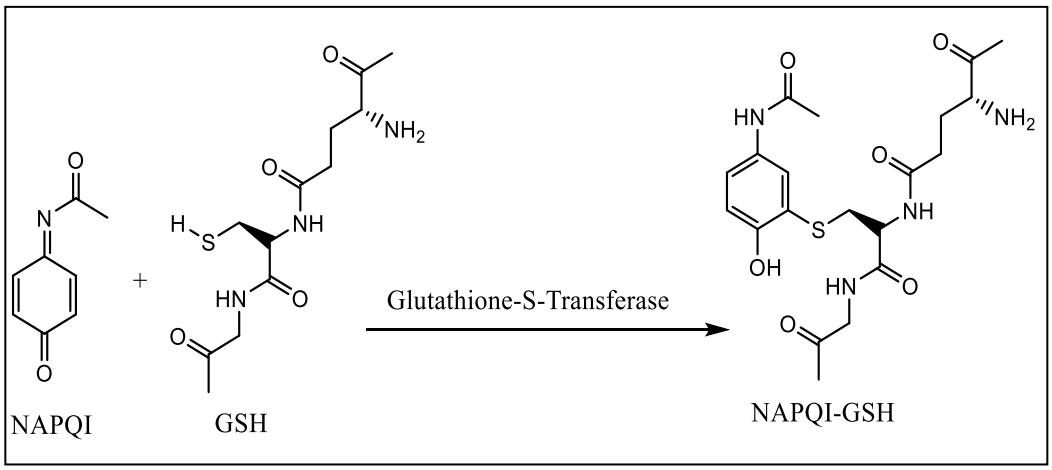

Figure 1. 9. Conjugation of NAPQI with GSH Catalyzed by Glutathione-S-Transferase.

CHAPTER II

EXPERIMENTS

II. A. Source of Purchased Materials

The 4-nitrophenol was purchased from Sigma-Aldrich, St Louis, MO, USA. The 4-nitrocatechol was purchased from Acros Organics, New Jersey, USA. The monobasic and dibasic potassium phosphates were purchased from Carolina Biology Supply Company, Burlington, North Carolina, USA.The reduced NADPH was purchased from Chem-Impex Int. Inc, Wood Dale, Illinois, USA. The 95% citral standard was purchased from Sigma-Aldrich, St Louis, MO, USA. The 98% reduced L-glutathione was purchased from Sigma-Aldrich, St Lois, MO, USA.The 98% 4-acetominophenol was purchased from Acros Organics, New Jersey, USA.The 99% eucalyptol was provided by Alfa Aesar, Ward Hill, Massachusetts, USA. The 99% coumarin was provided by Acros Organics, New Jersey, USA.

The rabbit liver cytochrome was prepared from frozen rabbit liver provided by Pel Freez Biological Inc, Arkansas, USA. The human liver cytochrome S9 fraction was from Sigma-Aldrich, St Louis, MO, USA. The purified human liver 2E1 enzyme was from Xeno Tech, Lenexa, KS, USA. The Human Liver Cytosol (Mixed Gender) was purchased

from Xeno Tech, Lenexa, KS, USA. The ginger rhizomes were originally grown and harvested in Costa Rican and Brazil. They were purchased from local markets in Greensboro, NC, USA.

II. A. i. Preparation of Ginger Extracts: Essential Oil and Hydrosol

The ginger rhizomes were processed in order to be ready for performing extraction by distillation. The ginger rhizomes were cleaned, peeled and sliced into uniform blocks with diameter of about 2mm. Then the ginger root blocks would be delivered into the flask of the formerly set up hydro-distillation equipment (Figure 2. 1) in a mass-volume proportion of 500g ginger root blocks / 1500mL DI water and distillated with DI water at 100 ˚C at 1atm for around 5 hours. The essential oil was collected from the oil phase in the upper layer of liquid in the collector and the hydrosol was from the water phase in the lower layer. The collected oil phase was centrifuged at 14000rpm for 10min to remove the attached water. Both of the essential oil and hydrosol were stored at -80 ˚C.

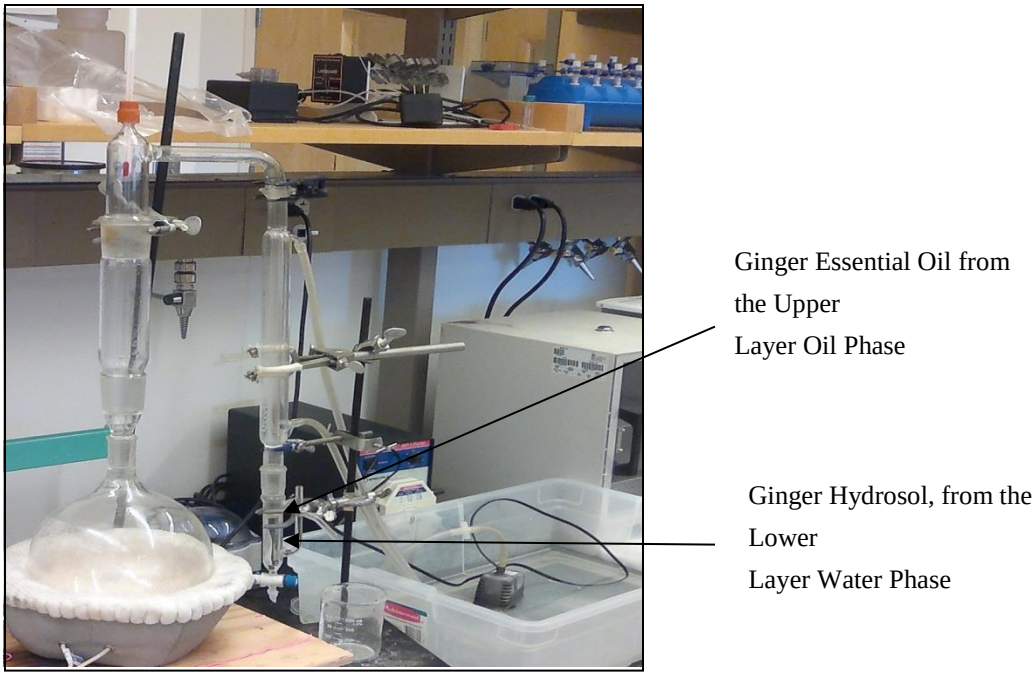

Figure 2. 1. Apparatus of Steam Distillation for Ginger Extract Production. The ginger essential oil was collected from the upper layer in the collector and the ginger hydrosol was collected from the lower layer in the collector.

II. B. Dose Response Tests of CYP 2E1 and CYP2A6 with Ginger Extracts

Dose responses tests of CYP2E1 and CYP2A6 were carried out with addition of ginger essential oil and hydrosol to assay the enzymes' activity changes and thus the inhibitory potencies of the two ginger extracts.

II. B. i. Dose Response Tests of CYP2E1 with Ginger Essential Oil and Hydrosol

The enzyme sources used in the dose response tests for CYP2E1 were liver S9 fraction from rabbit liver (Pel Freez Biological Inc, Arkansas, USA) and human liver

(Molecular Toxicology Inc.). The total volume of each reaction mixture was 200µL. The final concentration of CYP450 from rabbit liver was 40 nM and the final concentration of CYP450 from human liver was 33 nM. The buffer used in this assay was potassium phosphate buffer with a pH of 7.0 and a final concentration of 0.1Mfor each reaction. The substrate was 4-nitrophenolwith a dose of 60 µM for assays of rabbit liver CYP2E1 and 70 µM for assays of human liver CYP2E1.For the dose response test of ginger extracts, the ginger essential oil was added at doses of 6mg/L, 12mg/L, 18mg/L and 24mg/L and the ginger hydrosol was added in a in a volume percentage series of 12.5%, 25%, 37.5%, 50% in the reaction mixtures. After 8 minutes of pre-incubation at 37˚C, NADPH was added to initiate the reaction with a final concentration of 1.0mM. The reactions were incubated at37˚C for 45 minutes beforebeing quenched by 200µL 7% perchloric acid. Then each tube of mixture was be centrifuged at 14,000 rpm for 10 min and the supernatants were run on HPLC (Shimazu LC-20AT) to analyze the catalytic activity of human & rabbit liver CYP2E1. This analysis was carried out by monitoring the area of the optical density signal of the product 4-nitrocatechol at the wavelength of 340 nm (Figure 2. 2). The results are expressed as average percentage of enzyme activities± average percentage of standard deviations. To do this analysis, a C-18 column (4.6×150 mm) was used with a mobile phase of H2O/ acetonitrile/ trifluoroacetate (70/ 30/ 0.1 v/ v/ v) at a flow rate of 1.000 mL /min. The absorption wavelength for analysis was selected at 340 nm and the product peak

of 4-nitrocatecol was eluted at about 5.2 min. The peaks of the product were integrated and the dose response of the ginger hydrosol was measured as percent decrease of the product peaks with standard deviations.

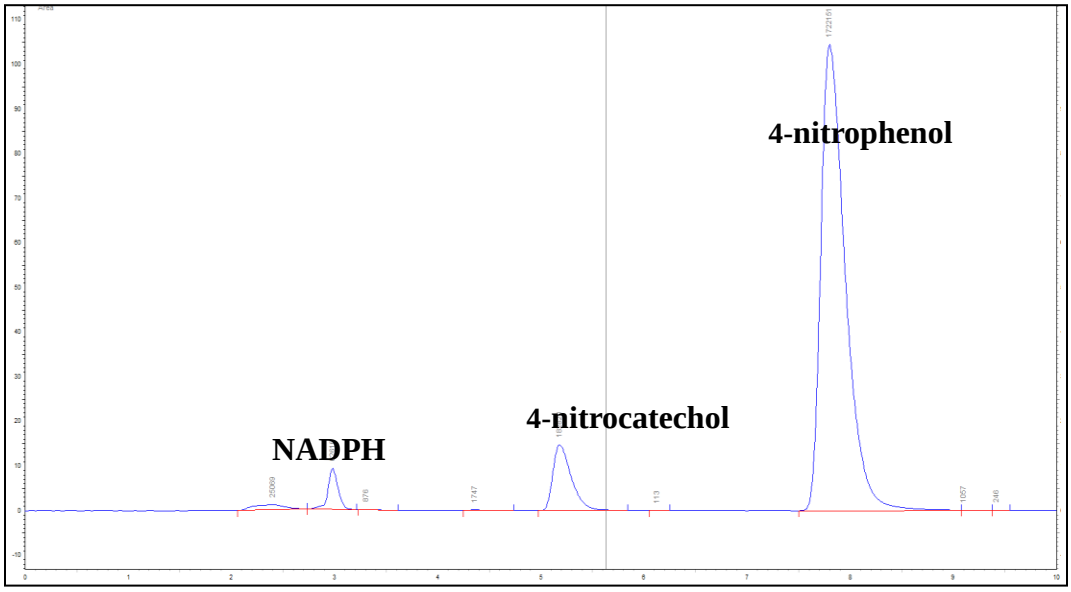

Figure 2. 2.The HPLC Chromatogram of the CYP 2E1 Assay. The chromatogram was obtained by the UV detector under the wavelength of 340nm.The product peak of 4-nitrocatecol was eluted at about 5.2 min.

II. B. ii. Dose Response Tests of CYP2A6 with Ginger Essential Oil and Hydrosol

The enzyme sources used in the dose response assays for CYP2A6 were S9 fraction from rabbit liver (Pel Freez Biological Inc, Arkansas, USA).Each reaction mixture was 200µL within which the concentration of CYP450 from rabbit liver was 40 nM and the CYP450 for human liver S9 fraction was 33 nM. The buffer that was used in this assay was also potassium phosphate buffer with pH of 7.0 and a final

concentration of 0.1M in each reaction mixture. The substrate used in the 2A6 assays was coumarin (1, 2-benzopyrone) with a final dose of 3μM each. For the dose response test of ginger extracts, the ginger essential oil was added at doses of 6mg/L, 12mg/L, 18mg/L and 24mg/L and the ginger hydrosol was added in a in a volume percentage series of 12.5%, 25%, 37.5%, 50% in the reaction mixtures. After 8 minutes of pre-incubation at 37˚C, NADPH was added to initiate the reaction with a final concentration of 1.0mM. The reactions were incubated at 37˚C for 30 minutes and quenched by 20 μL 70% perchloric acid. Then each tube of mixture was centrifuged at 14,000 rpm for 10 min and the supernatants were run on HPLC (Shimazu LC-20AT) to analyze the catalytic activity of human & rabbit liver CYP2A6.This analysis was carried out by monitoring the area of the optical density signal of the product of 7-hydroxycoumarin at the wavelength of 320 nm.The results are expressed as average percentage of enzyme activities± average percentage of standard deviations. To do this analysis, a C-18 column (4.6×150 mm) was used with a mobile phase of H_2O/ acetonitrile/ trifluoroacetate (70/ 30/ 0.1 v/ v/ v) at a flow rate of 1.000 mL / min. The absorption wavelength for analysis was selected at 320 nm and the product peak of 7-hydroxycoumarin was eluted at about 3.1 min as shown in Figure 2. 3. The peaks of the product were integrated and the dose response of the ginger hydrosol was measured as percent decrease of the product peaks with standard deviations.

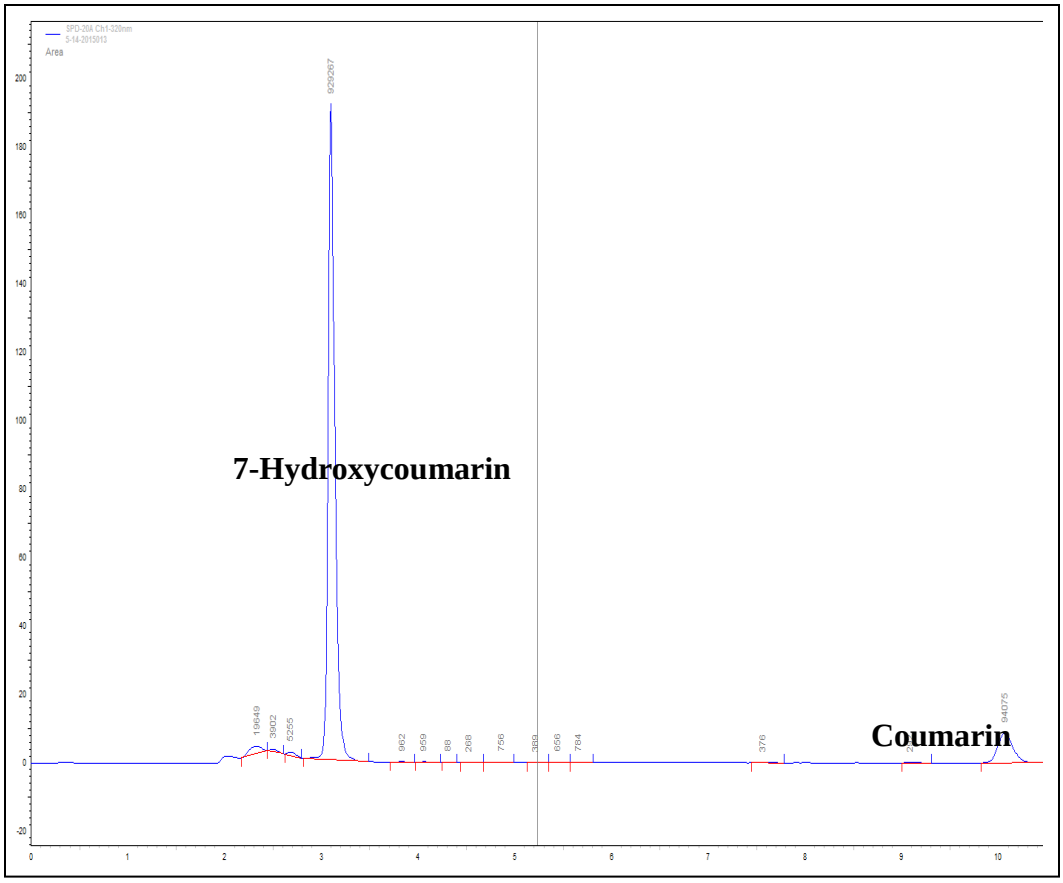

Figure 2. 3. The HPLC Chromatogram of the CYP 2A6 Assay. The chromatogram was obtained by the UV detector under the wavelength of 320nm.The product peak of 7-hydroxycoumarin was eluted at about 3.1 min.

II. C. Component Analysis of Ginger Extracts: Essential Oil and Hydrosol

The component analysis of the two ginger extracts, i. e. ginger essential oil and ginger hydrosol were performed in order to identify the possible inhibitors to CYP2E1 and CYP2A6.

II. C. i. Content Analysis of Ginger Essential Oils

The ginger essential oils from Brazilian ginger and Costa Rican Ginger were directly analyzed by GC mass spectrometer. For the GC mass spectrometry, the organic solvent used was hexane. Each oil sample was added with 5 µL in 200 µL of hexane before being run on GC-MS. The total elution time was 10.0 min.

II. C. ii. Component Analysis of Ginger Hydrosols

The organic components dissolved in the water phase of ginger hydrosol were trapped onto the C18 column (4.8×150 mm, Shimazu) with a mobile phase consisted of close to 100% DI water and 0.1%TFA by injecting 1500µL hydrosol in the HPLC. The trapped components were eluted with a mobile phase of 100% methanol and the eluted solution were analyzed by GC-MS (Shimazu SSI GCMS-QP2010S).

II. D. Dose Response Tests of CYP2E1 with Major Components from Ginger Hydrosol

After the major components of ginger hydrosol were determined, dose response tests were done in order to find the major components as the major inhibitors to the CYP2E1. The dose response tests started from assays with rabbit liver CYP2E1. Once a dose response was found to be attributed to a certain component, it would be tested in an assay with human liver CYP2E1. The setup for the dose response tests of CYP2E1 with components of ginger extracts was the same as for the assays done for testing the dose responses of CYP2E1 with ginger extracts. Because small chain alcohols were already

determined not to inhibit CYP2E1 by early projects of our group, no dose dependent assay was done of CYP2E1 with 2-heptanol. The dose response tests were only carried out with eucalyptol and citral. The major components of ginger hydrosol determined in this research were shown in Figure 2. 4.

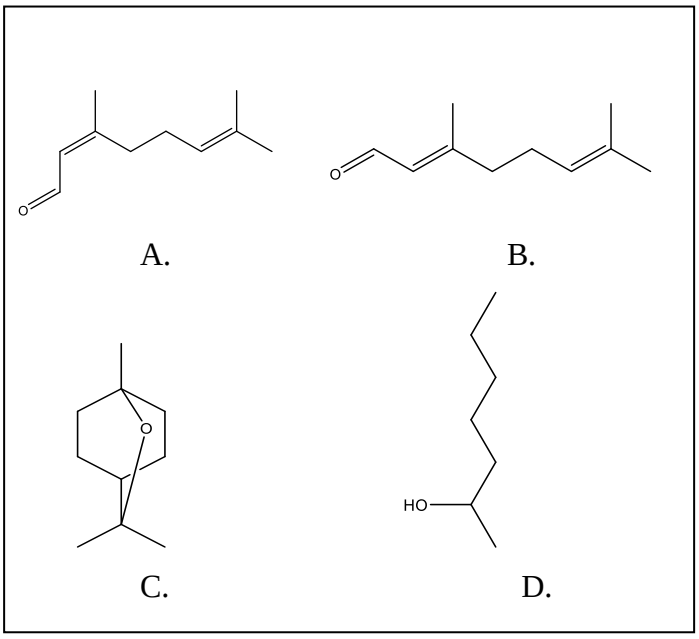

Figure 2. 4. Four Major Components of Ginger Hydrosol that Were Determined. A. B: Citral; A: (Z)-3.7-Dimethyl-2,6-octadienal (neral); B:E)-3,7-Dimethyl-2,6-octadienal (Geranial) C: Eucalyptol; D: 2-Heptanol.

For the dose response test of eucalyptol, the eucalyptol (Alfa Aesar,Ward Hill, Massachusetts, USA) was added at doses of 115.2 µM, 230.4µM, 460.8µMin the reaction mixtures. The reactions after incubation were analyzed by HPLC (ShimazuLC-20AT) under the same conditions as the assays for the dose response tests of CYP2E1 with ginger extracts.

For the dose response test of citral, the citral standard was 95% in purity with an isomer ratio of neral: geranial=1:1.8 (Acros Organics, New Jersey, USA). It was added at doses of 0, 112 µM, 245µM, 489µMin the reaction mixtures. The reactions after incubation were analyzed by HPLC (ShimazuLC-20AT) under the same conditions as the assays for the dose response tests of CYP2E1 with ginger extracts.

II. E. Standardization of Citral in Ginger Extracts

Standardization of citral concentrations in ginger extracts were carried out in order to know the concentrations of citral in each ginger extract. The ginger extracts, i.e. the Brazilian and Costa Rican ginger essential oil aqueous solution and hydrosol were run on HPLC (Shimazu LC-20AT) with a C18 column (Waters, 4.6 ×250mm) with a flow rate of 1.0mL/min and a mobile phase of 50% acetonitrile + 50% DI water + 0.1% TFA. The absorption wavelength was set as λ=260 nm. The two peaks of neral and geranial were eluted at 8.5 to 9.2 min and 9.2 to 9.9 min (Figure 2. 5). The first eluted peak was for neral and the second was for geranial, referring to the method of Sacks et al [27]. The concentrations of citral in the four ginger extracts were standardized with citral standard solutions.

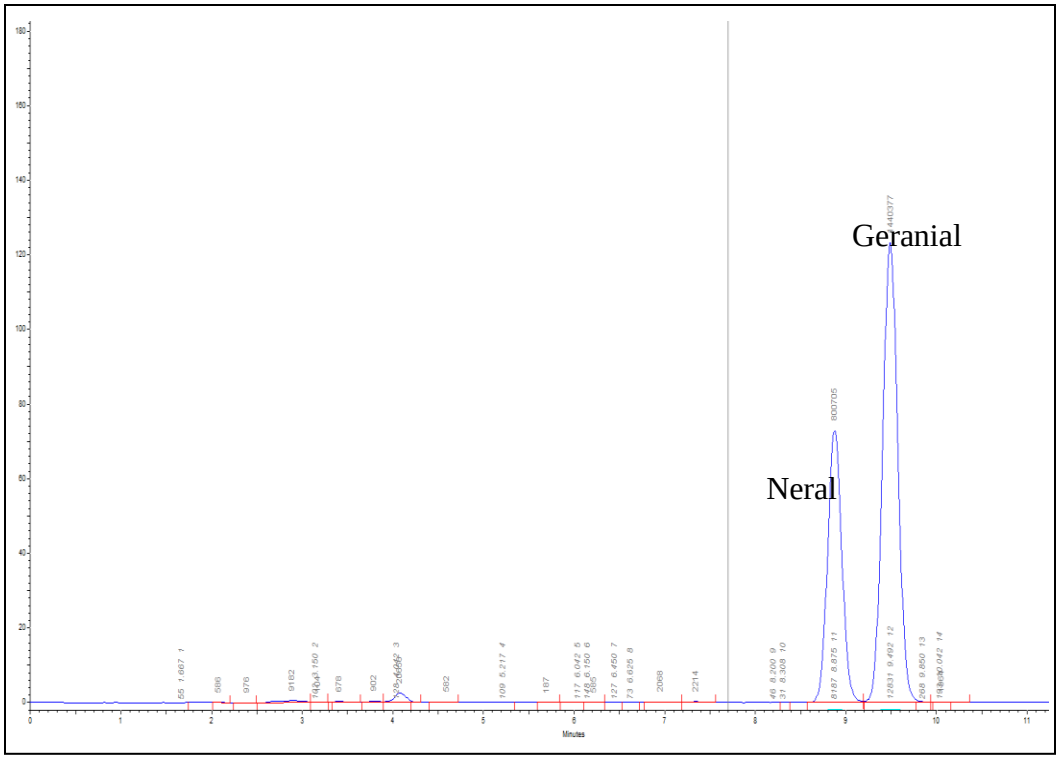

Figure 2. 5. HPLC Chromatogram of Netral and Geranial in Ginger Extracts. The ratio of the peak areas of neral geranial for the ginger extracts were calculated as the isomer ratio (neral to geranial) of all the ginger extracts.

II. F. Preparation of Standard Citral Solution with Desired Isomer Ratio

Standard citral solutions with a neral to geranial ratio close to 1:1.4 of ginger hydrosol should be made to assess the Michaelis-Menten kinetics of citral as a component from ginger hydrosol on human liver CYP2E1. Standard solution of citral with such an isomer ratio was obtained by isolation on HPLC (Shimazu LC20-AT) with a semi-prep column (HS-1010-M185). To prevent from disturbance of residue organic mobile phase on CYP2E1 in the assays, the dose response tests of an organic mobile

phase on human liver CYP2E1 were done to select one with an adequately low inhibition. Methanol and acetonitrile were tested in dose response assays with the same conditions as assays for dose response tests for ginger extracts. The only difference was that the acetonitrile was added at volume doses at 0.5%, 1% and 2% where as the methanol was added at 0.1%, 0.2%, 0.5% and 1%. The one with an adequately low inhibition on CYP2E1 was selected as the mobile phase for the preparation of citral with the desired isomer ratio. At the beginning of citral isolation, 20μL crude citral with a 95% purity (provided by Sigma-Aldrich) was trapped onto the semi-prep column with a mobile phase composed of 100% DI water and 0.1% TFA. Then the citral component were eluted by another TFA-free mobile phase of 90% DI water + 10% acetonitrile. Solutions with predominantly neral & geranial were obtained by collecting the HPLC elutes when the earlier and later parts of the peaks of citral showed up. The citral standard solution with neral to geranial ratio of 1:1.27 and 1:1.45 were obtained by mixing the solutions of neral and geranial to calculated volumetric proportions. These citral solutions were stored at –80˚C.

II. G. Michaelis-Menten Kinetic Analysis of Human Liver CYP2E1 with Citral or Ginger Extracts

After the dose responses of CYP2E1 to ginger extracts and isolated components of ginger extracts were tested, the Michaelis-Menten kinetic analysis of the ginger extracts

and the CYP2E1 inhibitory component(s) were carried out to determine their inhibitory kinetics. For the reaction mixtures the dose of enzymes (rabbit liver CYP2E1 and human liver CYP2E1), NADPH, buffer were set to be the same concentrations as that of the dose response assay of CYP2E1. The volume for each reaction mixture was still 200 µL and the time for incubation was still 45 min. Each reaction was carried out in triplicates. For the same curve of trial with inhibitor, the same dose of ginger extract was used for all the reactions. The citral used as inhibitor for human liver CYP2E1 and purified human liver 2E1 had an isomer ratio of neral : geranial =1:1.27. The concentrations of the substrate PNP in each incubation tube were set at 30µM, 60 µM, 120 µM, 240 µM, 480µM for the kinetic analysis with ginger extracts and were 15µM, 30µM, 60 µM, 120 µM, 240 µM for analysis with citral. The ginger extracts tested were the essential oil solution and hydrosol from both Costa Rican and Brazilian ginger roots. The Michaelis-Menten curves were generated with the software of Slide Write and the V_{max} and K_M were calculated. The types of inhibition, i.e. competitive/ un-competitive/ non-competitive were analyzed and the inhibitory constants of K_I for competitive inhibition and K_I' for non-competitive inhibition were calculated with the equations of $K_I=[I]*K_M/(K_M^{app}-K_M)$ and$K_I'=[I]*V_{max}^{app}/(V_{max}-V_{max}^{app})$.

II. H. Assessment of Inhibition of Human Liver CYP2E1 by Ginger Hydrosol in Acetaminophen Metabolism

Assays were designed and carried out to assess the potency of ginger hydrosol in the inhibition of the metabolism of acetaminophen by human liver CYP2E1. It started with synthesis of NAPQI-glutathione (NAPQI-GSH) via organic synthetic method to provide with the standard for analysis. Catalytic formation of NAPQI-GSH complex were set up with purified human liver CYP2E1 and GSH reductase from human liver cytosol and the identification of NAPQI-GSH were carried out with UPLC-MS. Finally, the dose response tests of citral in metabolism of acetaminophen by human liver CYP2E1 were carried out with the reaction of NAPQI-GSH's formation with presence of ginger hydrosol.

II. H. i. Synthesis of NAPQI-Glutathione Complex Standard

The method for synthesizing NAPQI-glutathione complex was described by Rousar T et al [28]. It consisted of the following steps.

Silver nitrate (4.224g) was dissolved in 65 mL DI water. 8g sodium hydroxide was dissolved in 250mL DI water. Silver oxide was generated by mixing both of the solutions above. Then the silveroxide formed was filtered by glass sinter glass funnel with vacuum,rinsed and stored.

4-Acetominophenol (Figure 2. 6) 0.428g was dissolved in chloroform dried with nitrogen gas. The newly made silver oxide was added in the chloroform solution of 4-nitrophenol and the mixture was stirred for 1 hour to generate NAPQI (Figure 2. 7). The mixture was filtered and the yellow chloroform solution containing NAPQI was stored.

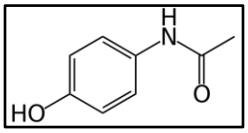

Figure 2. 6. Formula of 4-Acetaminophen: N-Acetyl-p-aminophenol (APAP).

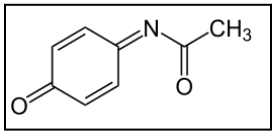

Figure 2. 7. Formula of N-Acetyl-p-Benzoquinone Imine (NAPQI).

Reduced glutathione was dissolved in 250 mL 0.1M sodium phosphate buffer with a pH of 7.4. The chloroform solution of NAPQI was added drop-wise in the glutathione solution while the glutathione solution was stirred to allow glutathione to conjugate with NAPQI. The water phase containing the complex was isolated by a separatory funnel. The water was evaporated with a rotary vacuum evaporator (Heidolph Instruments, Leiden, Netherlands) at 40˚C at the pressure of 1mbar, 300rpm, and the resultant residue was stirred with 700mL methanol. The resultant liquid was filtered with glass sinter

funnel to remove the remaining solid which was stored as the first part of residue for detection of the target product, NAPQI-GSH conjugate. The clear methanol solution was collected for vacuum evaporation on a rotary evaporator (Heidolph Labolutions, Leiden, Netherlands) at the pressure of 1mbar, 300rpm to get rid of the majority of methanol. Further drying with nitrogen gas was done on the residue to remove the rest of methanol. The solid residue left from this step was also stored for detection of NAPQI-GSH complex (Figure 2. 8).

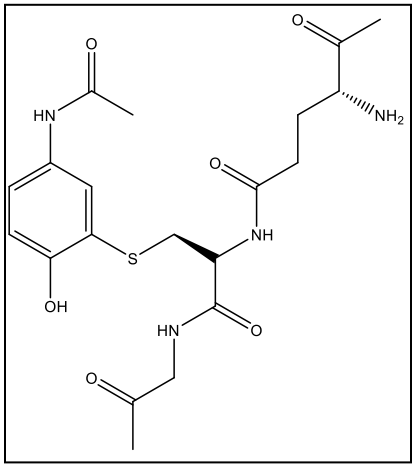

Figure 2. 8. Formula of NAPQI-GSH Complex.

The two parts of residues from the NAPQI-GSH generation step were both replenished with DI water to make their own stock solutions with the same mass concentration of 15g/ L. Both of these solutions were analyzed by the UPLC-MS (UPLC: Waters “Acuity Ultra Performance LC” with Waters C18 Column of 3.6×50mm; MS:

Thermo TSQ Quantum Access – Triple Stage Quadruple Mass Spectrometer) to identify the one in which the NAPQI-GSH was the most abundant. For the UPLC, the mobile phase was 5% acetonitrile + 95% DI water + 0.1% acetic acid and the flow rate was 0.3mL/min. The total run time was 10 min. The sample temperature was 10˚C and the column temperature was 40 ˚C. The ionization mode for the mass spectrometer was positive mode and the target molecules of NAPQI-GSH were protonated into ions with the mass of about 457.14 amu. The chromatograms for the protonated NAPQI-GSH were set to be expressed in the selected ions with the mass of 457.14 amu.

II. H. ii. Isolation of Standard NAPQI-GSH Complex from the Stock

After identifying the solid residue of which the aqueous solution contained the higher concentration of NAPQI-GSH complex at the mass concentration of 15g/ L, the solution of this residue was separated into five eluted fractions corresponding to the observed five isolated peaks by HPLC (Shimazu LC-20AT). These five fractions were analyzed to determine the fraction with the highest concentration of NAPQI-GSH complex and such a fraction would be used as the standard of NAPQI-GSH. The HPLC (Shimazu LC-20A, column: C18-4.6×250mm, Waters) was used to perform the separation with a mobile phase of 3% acetonitrile + 97% DI water + 0.1% TFA. The flow rate was 1.0mL/min. The components were separated and collected as five fractions as Fraction A at 2.5 min, Fraction B at 3.0min, Fraction C at between 3.5min and 4.0min, Fraction D at

between 4.5min and 5.0min and Fraction E at between 9min and 12min. These five fractions were analyzed by the UPLC-MS MS (UPLC: Waters "Acuity Ultra Performance LC" with Waters C18 Column of 3.6×50mm; MS: Thermo TSQ Quantum Access – Triple Stage Quadruple Mass Spectrometer) to identify the fraction with the most abundant NAPQI-GSH conjugate. The UPLC-MS in this step had the same parameters as used for identification of the major stock for synthesized NAPQI-GSH conjugate. This series of analysis was done for obtaining the HPLC fraction with the most abundant NAPQI-GSH content to be used as a standard for the assays in which NAPQI-GSH was the product of the reaction. The molecular mass of the target peak of ionized NAPQI-GSH was 457.14 amu.

II. H. iii. Setup of Enzyme Assay to Generate NAPQI-GSH

Each aliquot of reaction was 200µL with 0.1M sodium phosphate buffer with a pH of 7.0. The enzyme sources were purified human liver 2E1 and human liver cytosol (as source of glutathione-S-transferase). The concentration of 2E1 was 13.2µM and the cytosol used was 40µL in 200µL reaction mixture. As substrates, 4-acetominophenol was added at 2mM and reduced glutathione was added at2mM. Such high concentrations of the enzymes and the substrates were used to get a high-enough yield of target NAPQI-GSH conjugate so as to get a signal response that was high enough. For the reactions of positive trials, 1.0mM NADPH was added to start the reactions. In the group

of negative control, no NADPH was added to make contrast. (Before starting the reactions, the reaction mixtures without NADPH were pre-incubated at 37˚C for 10min.) After NADPH was added, the reaction mixtures were homogenized by vortex and incubated at 37˚C for 60min before being quenched with 200μL acetonitrile. The liquids were centrifuged at 14,000rpm for 10min before for LC-MS analysis. The parameters for the LC-MS were the same as that for the identification of NAPQI-GSH conjugate from the synthesized crude material.

II. H. iv. Dose Response Assay of Purified Human Liver CYP2E1 with Ginger Hydrosol

The total volume for each reaction was 200μLwith 0.1M sodium phosphate buffer with a pH of 7.0. Each reaction mixture had human liver CYP2E1 2.7nM together with human liver cytosol with a concentration of 5% of its original concentration as the source for glutathione-S-transferase. Reduced glutathione 2mM was used for all the reactions mixtures. The other substrate, acetaminophen, was tested at the concentration of800μM, which was close to the reported toxic concentration 1000μM [29]. NADPH was added to start the reactions. Brazilian ginger hydrosol was added at 12.5%, 25.0% and 50.0%. The peak for the protonated NAPQI-GSH with a molecular mass of around 457.14 amu appeared between 1.6 min and 2.2 min. The probable isomer of NAPQI-GSH conjugate was eluted between 0.6 min and 0.7 min. The peaks of the product (including the

isomerized product) were integrated and the dose response of the ginger hydrosol was measured as percent decrease of the product peaks. The dose responses were expressed in percentage of enzyme activity measured in peak areas of HPLC signal. Standard deviations were calculated.

II. I. Michaelis-Menten Kinetic Analysis of Rabbit Liver CYP2E1 with Ginger Extracts or Citral

The set-up and components of the reaction mixtures for Michaelis-Menten kinetic analysis for rabbit liver CYP2E1 were the same as that of human liver CYP2E1. The method for calculation of inhibitory constants was also the same. The only difference was that citral was added with three different ratios of isomers. Citral was tested at three isomer ratios. They were neral: geranial=1:1.27, neral: geranial=1:1.45, neral: geranial=1:1.8.

CHAPTER III

RESULTS AND DISCUSSION

III. A. Production of Ginger Extracts

The normal yield of the essential oil from ginger rhizome was about 2000 μL essential oil/ 500g ginger rhizomes. The ginger roots were frozen before being sliced for the convenience of processing. To prevent from loss of volatile constituents (observed as former dense smell of ginger in diffusing in the dryer box) and oxidation (observed as increased yellowish hue once compared with oil samples of the same source extracted without heated drying), the dehydration procedure was abandoned in order to procure the original volatile constituents of the ginger.

III. B. Dose Responses of CYP450 with Ginger Extracts

The dose response tests were done to assess the inhibitory effects of ginger essential oil and ginger hydrosol to the activities of the two isoforms of CYP450 enzymes: 2E1 and 2A6. The results were expressed in the remaining activities of enzyme with presence a series of different concentrations of ginger essential oil or hydrosol.

III. B. i. Dose Responses of CYP2E1 with Ginger Extracts

In comparison, both ginger essential oil and ginger hydrosol exhibited dose dependent inhibition on rabbit liver CYP2E1. For example, at the dose of 18 mg/L Costa Rican ginger essential oil, around 37.0% inhibition of activity was observed. At the dose of 18 mg/L Brazilian ginger essential oil, around 43.4% inhibition of activity was observed. At the dose of 12.5% of Costa Rican ginger hydrosols, around 66.2% inhibition of activity was observed. At the dose of 12.5% of Brazilian ginger hydrosol, around 65.5% inhibition of activity was observed (Figure 3. 1). Adding the ginger hydrosol had stronger inhibition to rabbit liver CYP2E1 than adding the same volume of 18mg/L aqueous solution of ginger essential oil. In comparison, the inhibition of ginger extracts on human liver CYP2E1 complied well with that on rabbit liver CYP2E1, with both ginger essential oil and ginger hydrosol showing dose dependent inhibition. For example, at the dose of 12 mg/L Costa Rican ginger essential oil, around 18.3% inhibition of activity was observed. At the dose of 12 mg/L Brazilian ginger essential oil, around 22.8% inhibition of activity was observed. At the dose of 12.5% of Costa Rican ginger hydrosol, around 44.8% inhibition of activity was observed. At the dose of 12.5% of Brazilian ginger hydrosol, around 60.0% inhibition of ginger hydrosol was observed. The hydrosol was also shown to give more prominent inhibition in enzyme assay of human liver CYP2E1 (Figure 3. 2).

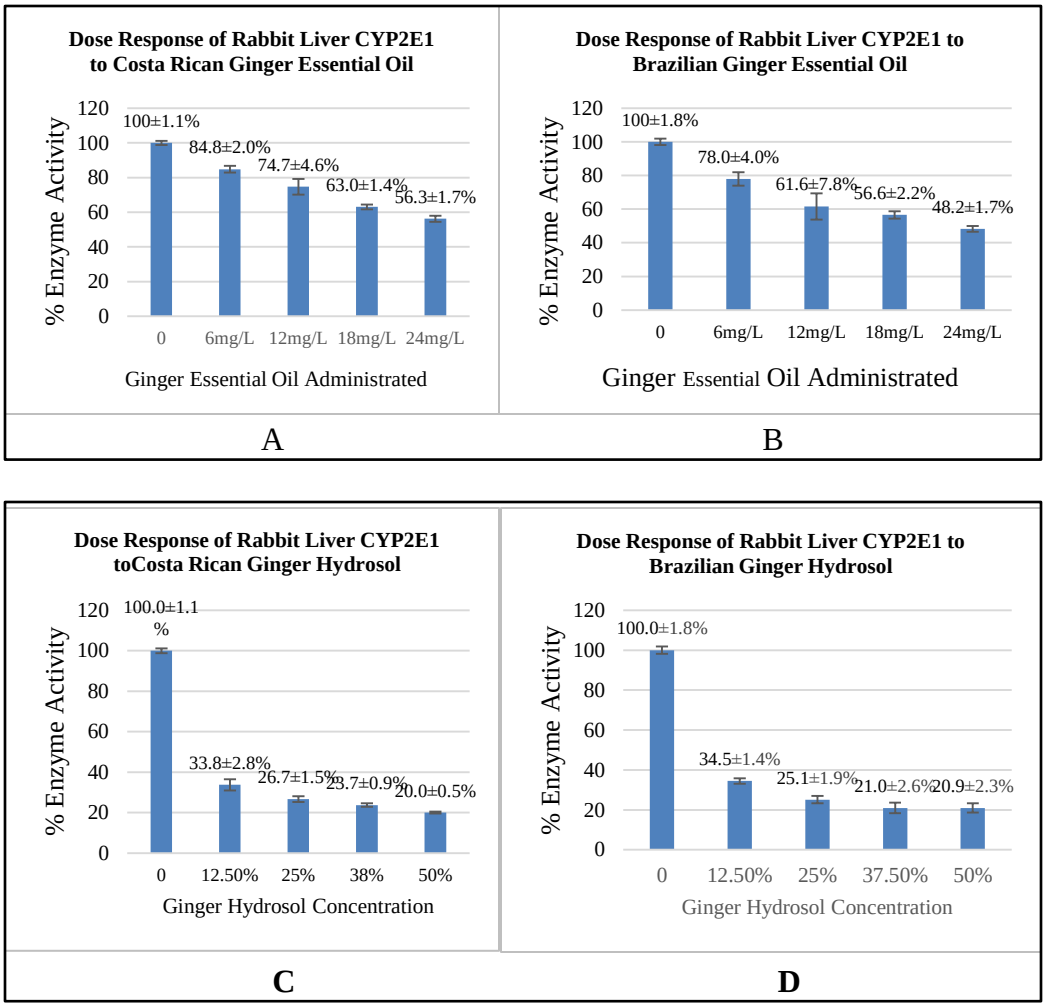

Figure 3. 1. Dose Responses of Rabbit Liver CYP2E1 to Ginger Extracts. **A.** Costa Rican ginger essential oil; **B.** Brazilian ginger essential oil; **C.** Costa Rican Ginger Hydrosol **D.** Brazilian ginger hydrosol.

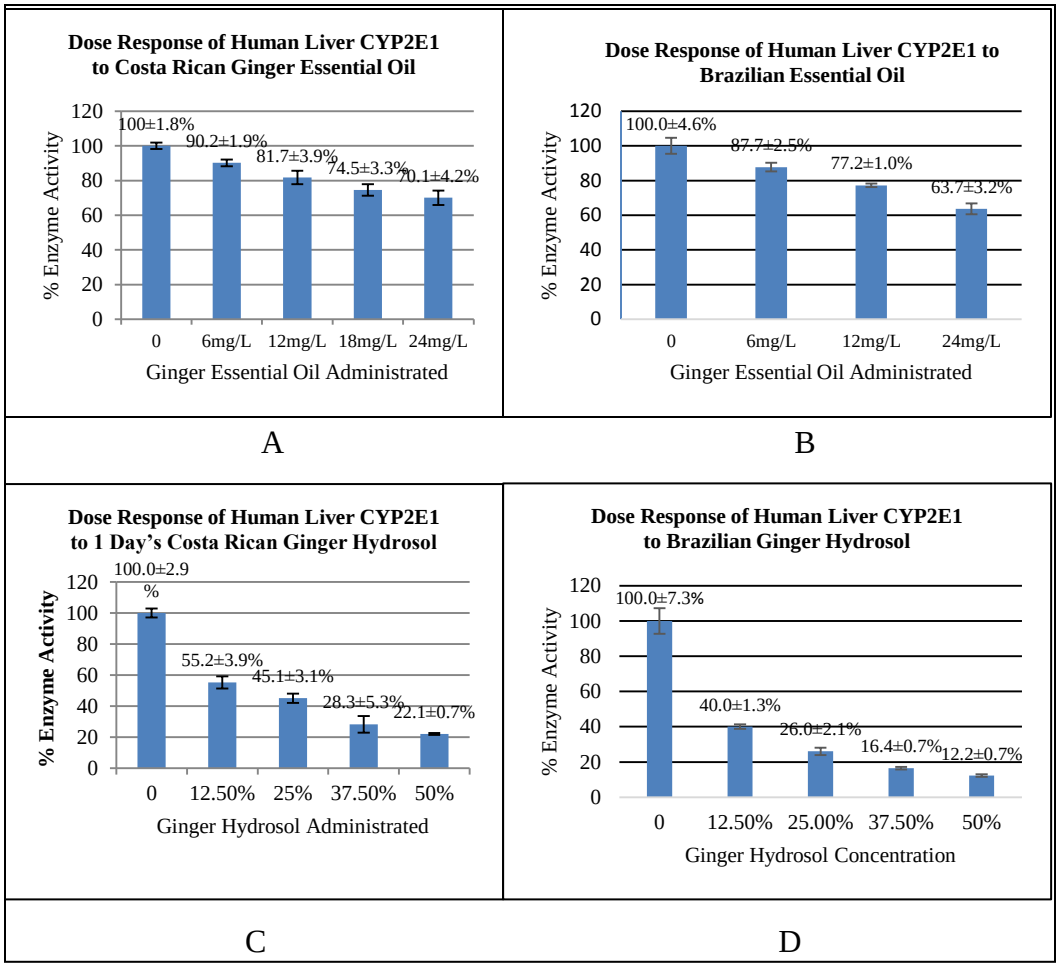

Figure 3. 2. Dose Responses of Human Liver CYP2E1 to Ginger Extracts. **A.** Costa Rican ginger essential oil; **B.** Brazilian ginger essential oil; **C.** Costa Rican ginger hydrosol **D.** Brazilian ginger hydrosol.

III. B. ii. Effect of Ginger Extracts on CYP2A6

In addition to CYP2E1, the ginger extracts were tested on CYP2A6 in order to determine the comparative effects of these two isoforms of CYP450 enzymes. In contrast to the dose responses of CYP2E1 to ginger extracts, the rabbit liver CYP2A6 showed poor dose responses to the presences of ginger essential oil (Figure 3. 3) and ginger

hydrosol (Figure 3. 4). These results implicated the same poor dose responses of human liver CYP2A6 to the ginger extracts. For example, at the dose of 50% Costa Rican ginger hydrosol, only around 16.3% inhibition of human liver CYP2A6 activity was observed. In contrast, at the dose of 50% Costa Rican ginger hydrosol, around 78.9% inhibition of activity of human liver CYP2E1 was observed (Figure 3. 2. C). Therefore, ginger extracts showed a more selective inhibition on CYP2E1.

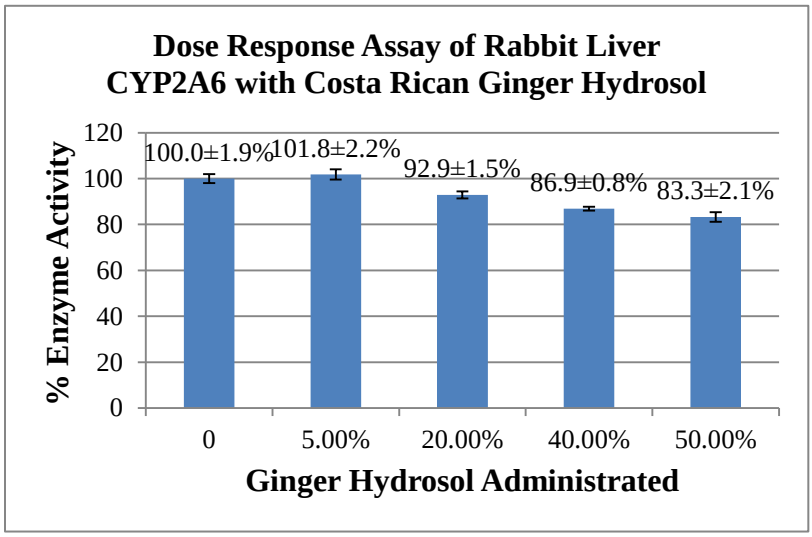

Figure 3. 3. Dose Response Test of Rabbit Liver CYP2A6 to Costa Rican Ginger Hydrosol. Only very weak inhibition was observed.

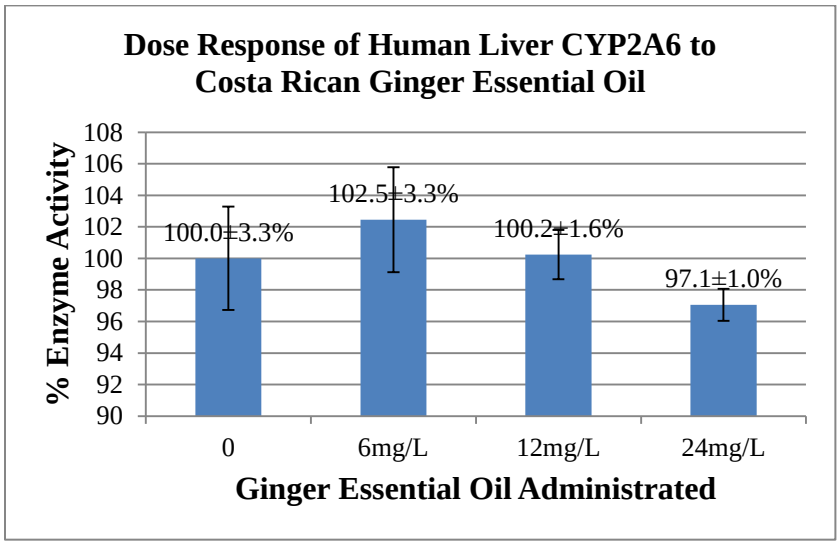

Figure 3. 4. Dose Response Test of Rabbit Liver 2A6 Assay to Ginger Essential Oil. The result indicated poor inhibition by ginger essential oil on CYP2A6.

III. C. Components of Ginger Extracts

The components of the samples of ginger essential oil were identified via direct analysis using GC-MS. The component analysis of the ginger hydrosols was done by firstly trapping the components onto a C18 column of HPLC, then eluting these components into methanol and finally analyzing these dissolved components by GC-MS. The results were expressed in the first part as the GC chromatograms of the organic components constituting the ginger essential oils and hydrosols. The second part was the bar graphs of the identified components in which the height of the bar of each component was proportional to its peak area in the corresponding GC chromatogram.

III. C. i. Components of Ginger Essential Oils

The components of ginger essential oils were shown in their GC-MS chromatograms together with bar graphs. For both samples of Costa Rican ginger essential oil and Brazilian ginger essential oil, there were five major peaks shown by the GC chromatograms shown by Figure 3. 5 and Figure 3. 6, respectively. The compositions of the two ginger essential oil samples were very similar. The chromatograms showed they both contained citral, which had two isomers of neral and geranial. The peak area percentages of neral and geranial for Brazilian ginger essential oil were 9.98% and 17.68%, whereas the percentages of neral and geranial for Costa Rican ginger essential oil were 5.50% and 9.21%. Both of the two essential oil samples contained camphene, of which the peak area percentages were 4.38% for Brazilian ginger essential oil and 9.42 for Costa Rican ginger essential oil. The data of the percentages of the components' peak areas were shown in Figure 3. 7 and Figure 3. 8.

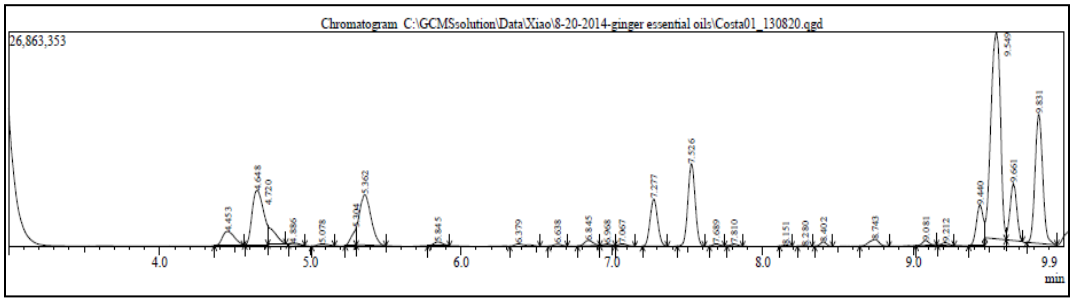

Figure 3. 5. GC Chromatogram of Costa Rican Ginger Essential Oil.

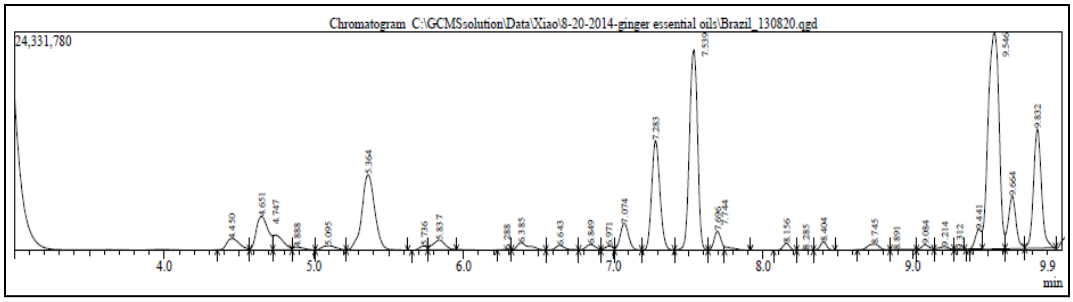

Figure 3. 6. GC Chromatogram of Brazilian Ginger Essential Oil.

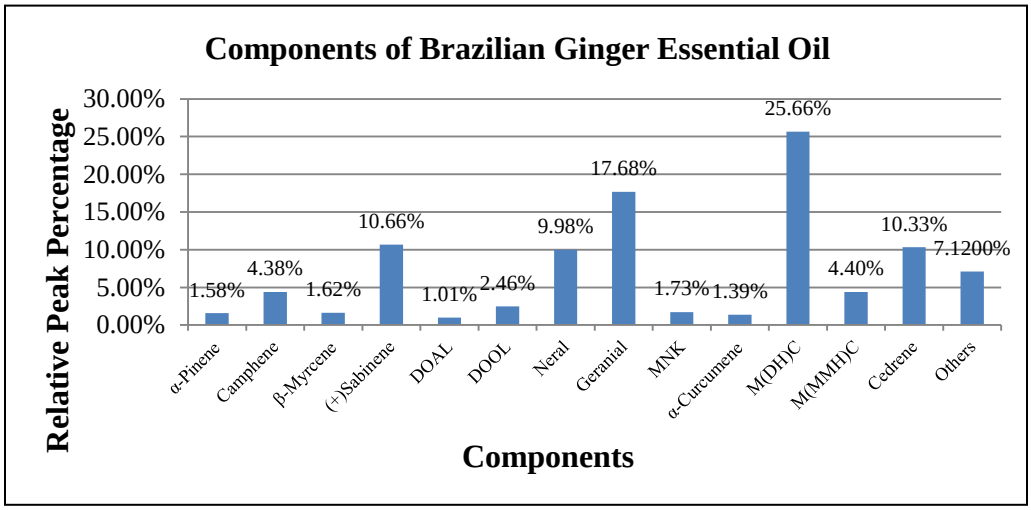

Figure 3. 7. Components of Brazilian Ginger Essential Oil. DOAL: 3, 7-dimethyl-6-octenal; MNK: methyl nonyl ketone; M(DH)C:2-methyl-5-(1,5-dimethyl-4-hexenyl)-1,3-cyclohexadiene; M(MMH)C:1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-(s)-cyclohexene

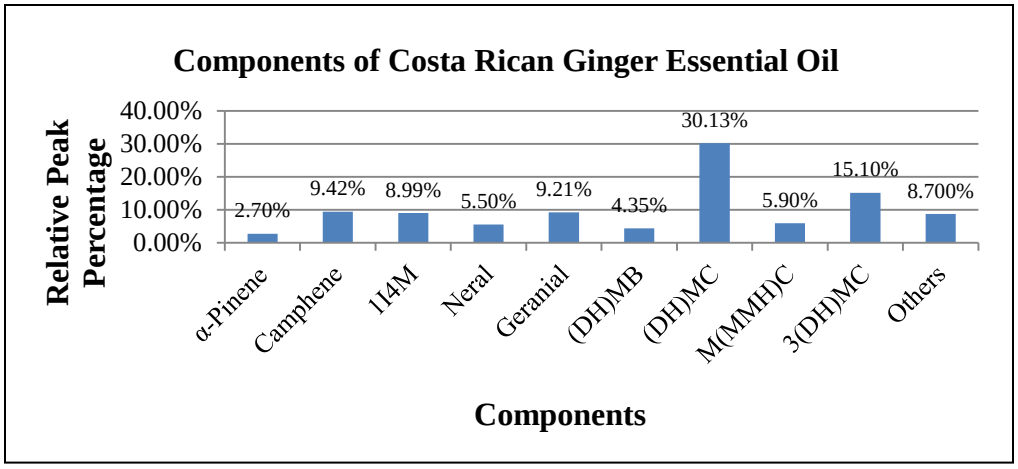

Figure 3. 8. Components of Costa Rican Ginger Essential Oil.
1I4M:1-isopropyl-4-methylenebicyclohexane; (DH)MB: 1-(1,5-dimethyl-4-hexenyl)-4-methyl benzene; (DH)MC:5-(1,5-dimethyl-4-hexenyl)-2-methyl-1,3-cyclohexadiene;
M(MMH)C: 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-cyclohexene;
3(DH)MC:3-(1,5-dimethyl-4-hexenyl)-6-methylene-1-cyclohexene

III. C. ii. Components of Ginger Hydrosols

The components of ginger hydrosols were shown in their GC-MS chromatograms together with bar graphs. For both samples of Costa Rican ginger hydrosol and Brazilian ginger hydrosol, there were five peaks shown by the GC chromatograms shown by Figure 3. 9 and Figure 3. 10, respectively. The components of the two ginger hydrosol samples were very similar. The major components of ginger hydrosol (with a peak area taking up ≥10% of the chromatogram) were 2-heptanol, eucalyptol and citral (with two isomers of neral and geranial) as shown in Figure 3. 11 and Figure 3. 12.

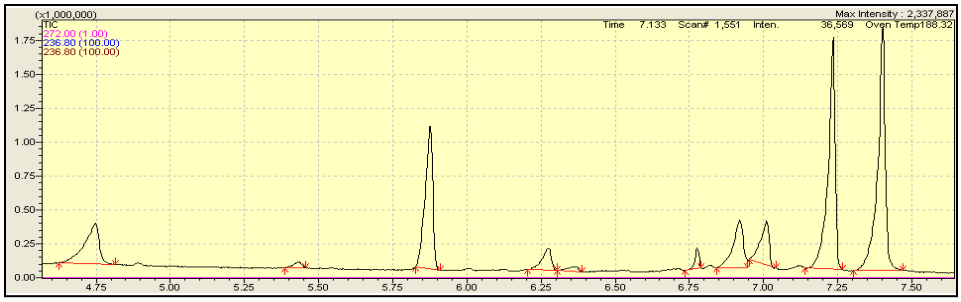

Figure 3. 9. GC Chromatogram of Costa Rican Ginger Hydrosol.

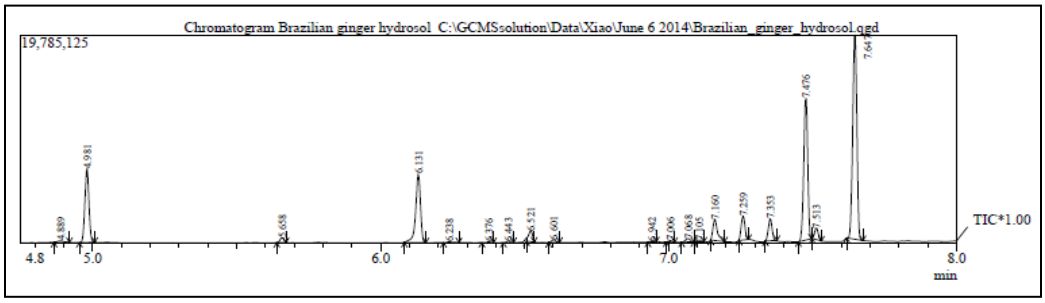

Figure 3. 10. GC Chromatogram of Brazilian Ginger Hydrosol.

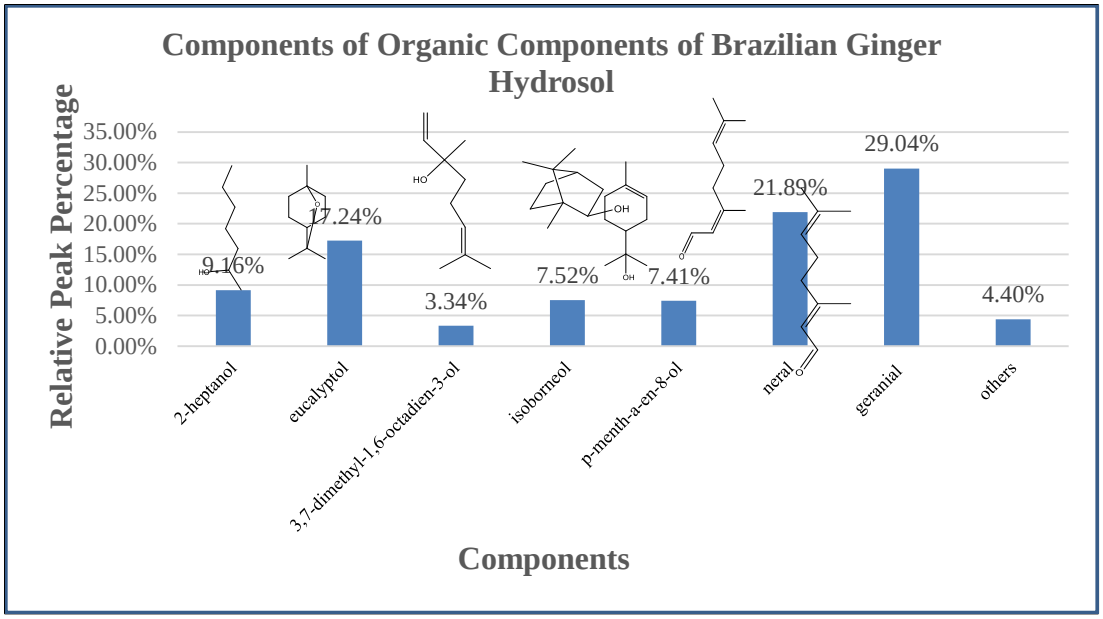

Figure 3. 11. Components of Costa Rican Ginger Hydrosol. The components of the ginger hydrosol were determined to be 2-heptanol (9.16%), eucalyptol (17.24%), 3,7-dimethyl-1,6-octadien-3-ol (3.34%), isoborneol (7.52%), p-menth-1-en-8-ol (7.41%), neral (24.5%), geranial (37.5%), others (4.40%).

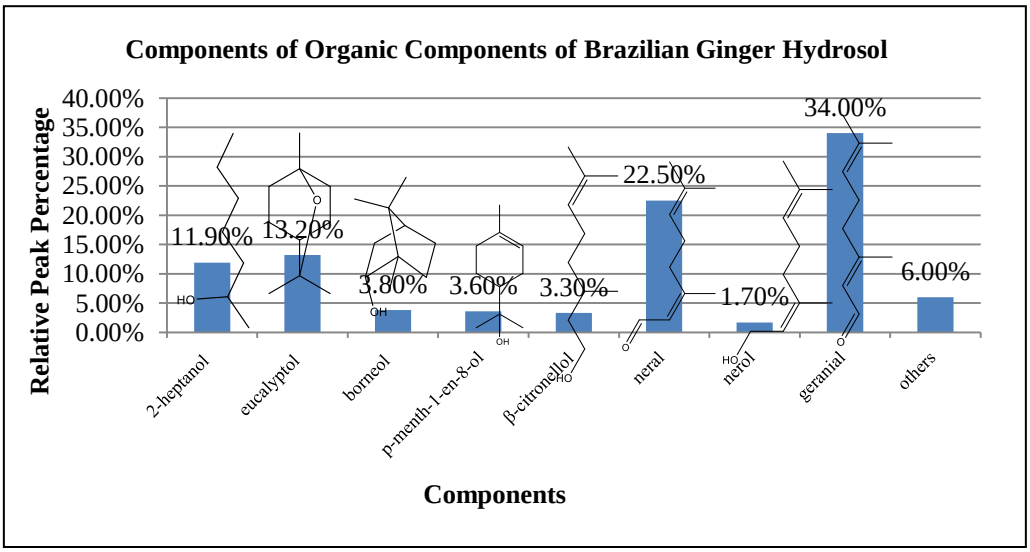

Figure 3. 12. Components of Brazilian Ginger Hydrosol. The components of the ginger hydrosol were determined to be 2-heptanol (11.9%), eucalyptol (13.2%), borneol (3.8%), p-menth-1-en-8-ol (3.6%), β-citronellol (3.3%), neral (22.5%), geranial (34.0%), others (6.0%).

III. D. Dose Response Tests of CYP2E1 with Major Components from Ginger Hydrosol

Because eucalyptol was determined as a major component of ginger hydrosols (14.2% for Costa Rican ginger hydrosol and 13.2% for Brazilian ginger hydrosol), it was tested in dose response assays of CYP2E1. It only showed weak inhibition at very high concentrations (Figure 3. 13). Therefore, eucalyptol was excluded as an inhibitor from ginger hydrosol to CYP2E1. In contrast, citral showed significant dose dependent inhibition on human liver CYP2E1. Greater than 50% inhibition of activity was observed at 100 μM concentration (Figure 3. 14).These results implicated citral as the target inhibitor for the further studies.

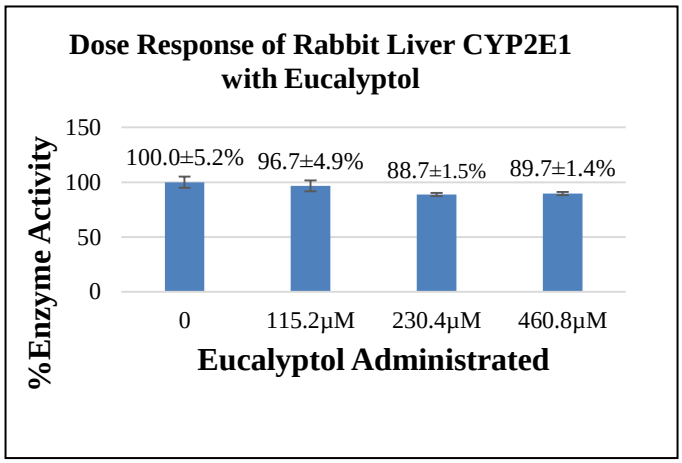

Figure 3. 13. Dose Response of Human Liver CYP2E1 to Eucalyptol. The dose dependent inhibition of eucalyptol on CYP2E1 was very weak.

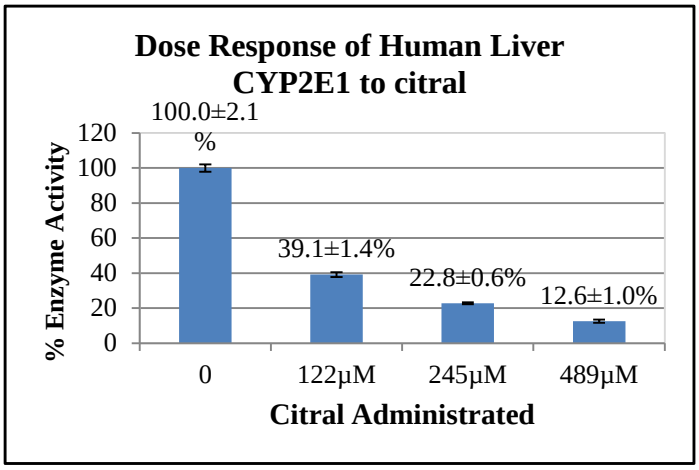

Figure 3. 14. Dose Response Test of Human Liver CYP2E1 to Citral Standard (Neral: Geranial=1:1.8). Citral showed remarkable inhibition on CYP2E1 at a dose of 122μM.

III. E. Standardization of Citral in Ginger Hydrosol

Because citral was determined as a major inhibitor in ginger hydrosol, its concentrations were standardized. For the hydrosol of Costa Rican ginger hydrosol, the citral's concentration was determined to be 965μM. For the hydrosol of Brazilian ginger, the citral's concentration was determined to be 1259μM. For both of the ginger hydrosols, the isomer ratio of citral was neral: gereanial=1: 1.4. In order to assess the contribution of citral to ginger extracts, the citral concentrations in ginger essential oil solutions and ginger hydrosols were standardized. For the ginger hydrosols, the citral concentrations were in the range of 1.0 mM. For the ginger essential oil solutions with a concentration of 48 mg/L, the citral concentrations were in the range of 70 to 90 μM as shown in Table 3. 1.

Table 3. 1. Standardization of Citral in Ginger Essential Oil and Hydrosol. For ginger essential oils, the ratio of neral: geranial=1: 1.8. For ginger hydrosols, the ratio of neral: geranial=1: 1.4. The concentration of citral in the ginger extracts, i.e. ginger essential oil's solution of 48mg/ L and original ginger hydrosol was standardized by HPLC (Shimazu LC20-AT).

Ginger Extracts	Citral Concentration	Ratio of Neral: Geranial
Costa Rican Ginger Hydrosol	965μM	1: 1.4
Brazilian Ginger Hydrosol	1259μM	1: 1.4
Costa Rican Ginger Essential Oil Solution: 48mg/L	73μM	1:1.8
Brazilian Ginger Essential Oil Solution: 48mg/L	91μM	1:1.8

III. F. Preparation of Standard Citral Solution with Desired Isomer Ratio

To prepare standard citral solution, dose response tests of CYP2E1 with organic solvents as mobile phases to elute citral were carried out to select the one with the weakest inhibitory potency to CYP2E1. For the selection of the organic mobile phase for isolation of citral standard, the results of dose dependence tests of human liver CYP2E1 showed stronger inhibition by methanol under the same conditions of the dose dependence tests of CYP2E1 with ginger extracts (Figure 3. 15). Therefore, acetonitrile was selected as the non-polar mobile phase for isolating citral stock solution with an isomer ratio that was similar with the isomer ratio of citral in ginger hydrosol. The isolated citral solutions contained citral with a concentration high enough and could be

sufficiently diluted to decrease the concentration of acetonitrile to as low as 0.1% before used in assays of CYP2E1. Therefore, the undesired effect of acetonitrile was removed. One fraction of isolated citral solution contained an isomer ratio of neral: geranial=1:1.27; it showed ~70% inhibition at 0.2mM to the purified human liver 2E1 (Figure 3. 16**)**. This dose response complied well with the results of human liver CYP2E1 in the S9 extract from human liver. It was noticed that the doses of enzyme administered did not affect the IC50 value of the purified CYP2E1 when citral was added as the 2E1 inhibitor, though the enzyme sources administered with a higher dose did show higher catalytic activities.

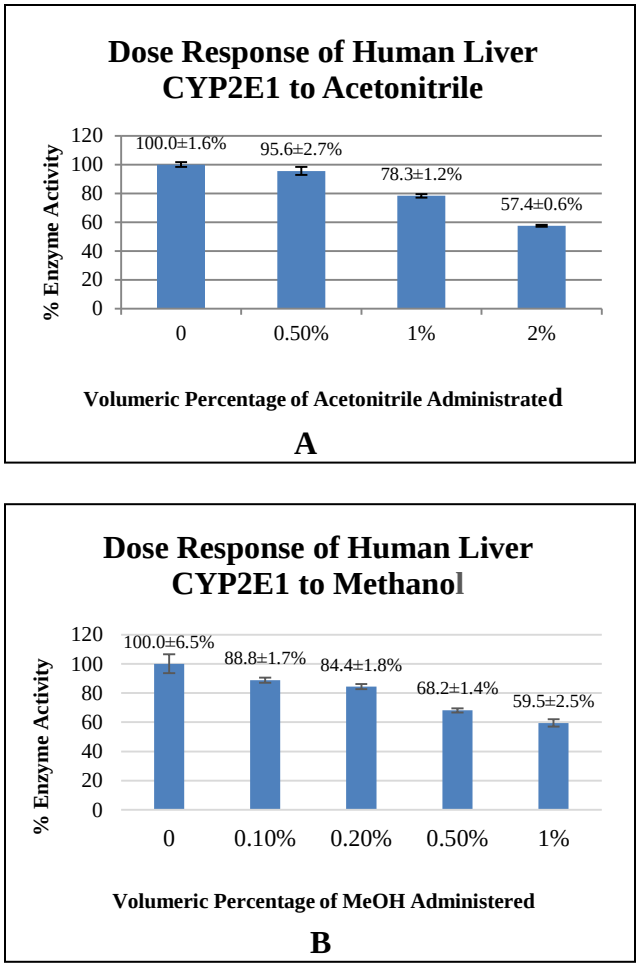

Figure 3. 15. Dose Responses of Human Liver CYP2E1 to Acetonitrile and Methanol. A. Dose Response to Acetonitrile; B. Dose Response to Methanol.

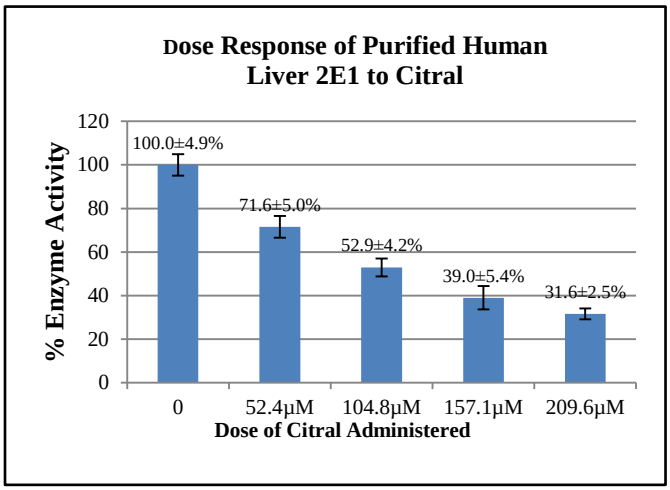

Figure 3. 16. Dose Response of Purified Human Liver CYP2E1 to Citral (Neral: Geranial=1:1.27). For each reaction, 42.5µM CYP450 was used.

III. G. Michaelis-Menten Kinetics of Human Liver CYP2E1 with Citral or Ginger Extracts

Michaelsis-Menten kinetic analysis of human liver CYP2E1 was carried out with or without presence of the inhibitors, i. e. citral or ginger extracts. The values (expressed with standard deviations) of K_M and V_{max} were shown in the curves of the enzyme without presence of inhibitors. The K_M^{app}, V_{max}^{app} values (expressed with standard deviations) were shown in the curves with presence of the inhibitors. The curves were shown with the doses of inhibitor added in the corresponding reactions. The results showed that citral (Neral: Geranial=1:1.27) was a competitive inhibitor to human liver CYP2E1 with an inhibitory constant K_I of 43.0µM. In comparison, both the ginger essential oil and ginger hydrosol were also determined as competitive inhibitors. For the

competitive inhibition, the inhibitory constant K_I was calculated with the equation of $K_I=K_M*[I]/ (K_M^{app}-K_M)$; for the non-competitive inhibition, the inhibitory constant KI' was calculated with the equation of $K_I'=[I]*V_{max}^{app}/ (V_{max}-V_{max}^{app})$. For the Brazilian ginger essential oil, its competitive inhibitory constant was 27.3mg/L:

$K_I= K_M*[I]/ (K_M^{app}-K_M)$=18.4mg/L*70.5 μM /(118.2 μM -70.5 μM)= 27.3mg/L.

The competitive inhibitory constant for Costa Rican ginger essential oil was calculated to be K_I=29.9mg/L. Meanwhile, the Brazilian ginger hydrosol showed a K_I that corresponding to 4.6% of its original concentration. Such a dose of hydrosol would contain 45.5 μM citral (neral: geranial=1:1.4) as referred to the standardized citral concentrations ginger hydrosols: 4.6% Brazilian ginger hydrosol=4.6%*1259μM citral=45.5μM citral. The K_I for the Brazilian ginger hydrosol was 3.6% of its original concentration. At this concentration, the citral's content was 44.3μM (neral: geranial=1:1.4). These data above were shown in Figure 3. 17 and Table 3. 2. These citral concentrations that correlated with Costa Rican ginger hydrosol's K_I and Brazilian ginger hydrosol's $K_I$in human liver CYP2E1 inhibition were very similar with the K_I of pure citral with a similar isomer proportion in the inhibition of the same human liver CYP2E1. This close similarity implicated that citral was the predominant CYP2E1 component in these ginger hydrosols.

Prior researches had shown citral as an inhibitor to other species of CYP450. For example, citral was shown by Raner et al as an inhibitor of CYP1A2 [30] and by Seo et al as an inhibitor of CYP2B6 [31]

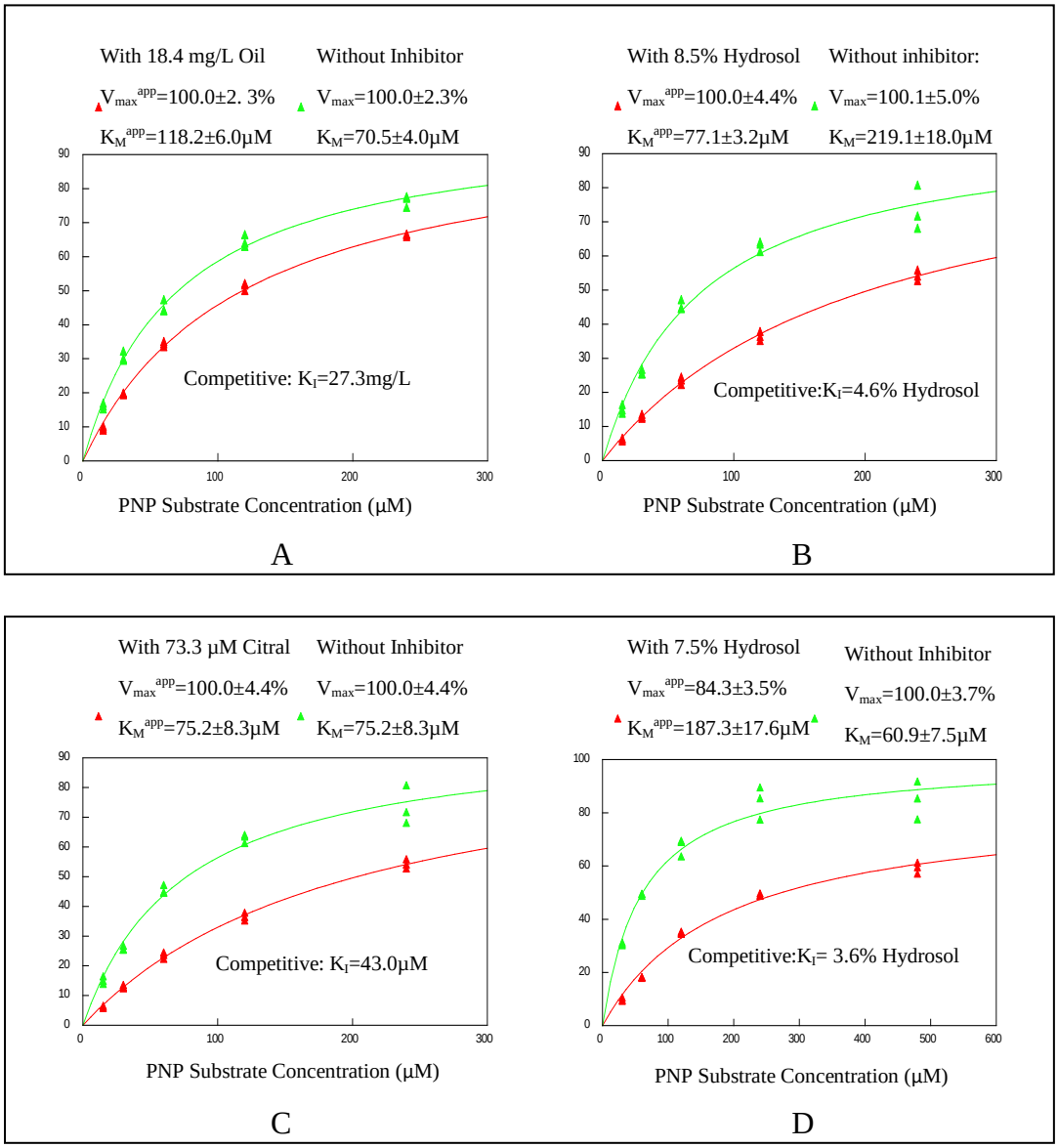

Figure 3. 17. Michaelis-Menten Curves of Human Liver CYP2E1 with Ginger Extracts.

A. Michaelis-Mentencurve with Brazilian ginger essential oil. **B**. Michaelis-Mentencurve with Costa Rican ginger hydrosol. **C**. Michaelis-Mentencurve with Citral (neral: geranial=1:1.27). **D**. Michaelis-Mentencurve with Brazilian Ginger Hydrosol.

Table 3. 2. Michaelis-Menten Kinetics of Human Liver CYP2E1 with Ginger Extracts and Citral Standard (Neral: Geranial=1:1.27).

Inhibitor	Neral: Geranial	Type of Inhibition	Inhibitory Constant
Citral	1:1.27	Competitive	43.0μM
Brazilian Ginger Essential Oil	1:1.8	Competitive	27.3mg/L
Costa Rican Ginger Essential Oil	1:1.8	Competitive	21.9mg/L
Brazilian Ginger Hydrosol	1:1.4	Competitive	3.6% of Original Concentration (45.5μM citral)
Costa Rican Ginger Hydrosol	1:1.4	Competitive	4.6% of Original Concentration (44.3 μM citral)

III. H. Inhibition of Human 2E1 by Ginger Hydrosol in Acetaminophen Metabolism

The synthesized standard of NAPQI-GSH was further isolated from the crude product of synthesized and the time for it to be eluted in the UPLC-MS chromatogram was determined. With the reference provided by the NAPQI-GSH standard, the peak and the time for elution of the NAPQI-GSH formed from catalytic reaction were determined. Ultimately, the inhibitory potency of ginger hydrosol to the activity of human liver CYP2E1 was determined.

III. H. i. Identification of the Stock of Synthesized NAPQI-Glutathione Complex

The LC-MS chromatograms showed that the component analysis of the two residues collected from the synthesis of NAPQI-GSH conjugate. From the peak area values (Figure 3. 18), the concentration of NAPQI-GSH in the residue solution from the evaporation of methanol was much higher than the concentration of NAPQI-GSH in the residue solution from the methanol extraction. At the same mass concentrations of 15 g/ L, the aqueous solution of the solid residue collected from condensation by methanol evaporation showed a signal peak of NAPQI-GSH with an area 76 times more than the peak area for the solution of residue from methanol extraction. Therefore, stock solution of this residue from condensation by methanol evaporation was used as the source for isolating the standard of NAPQI-GSH conjugate to detect the NAPQI-GSH formation in the assays in which the formation of NAPQI-GSH was catalyzed by CYP2E1 and glutathione-S-transferase contained by the human liver cytosol.

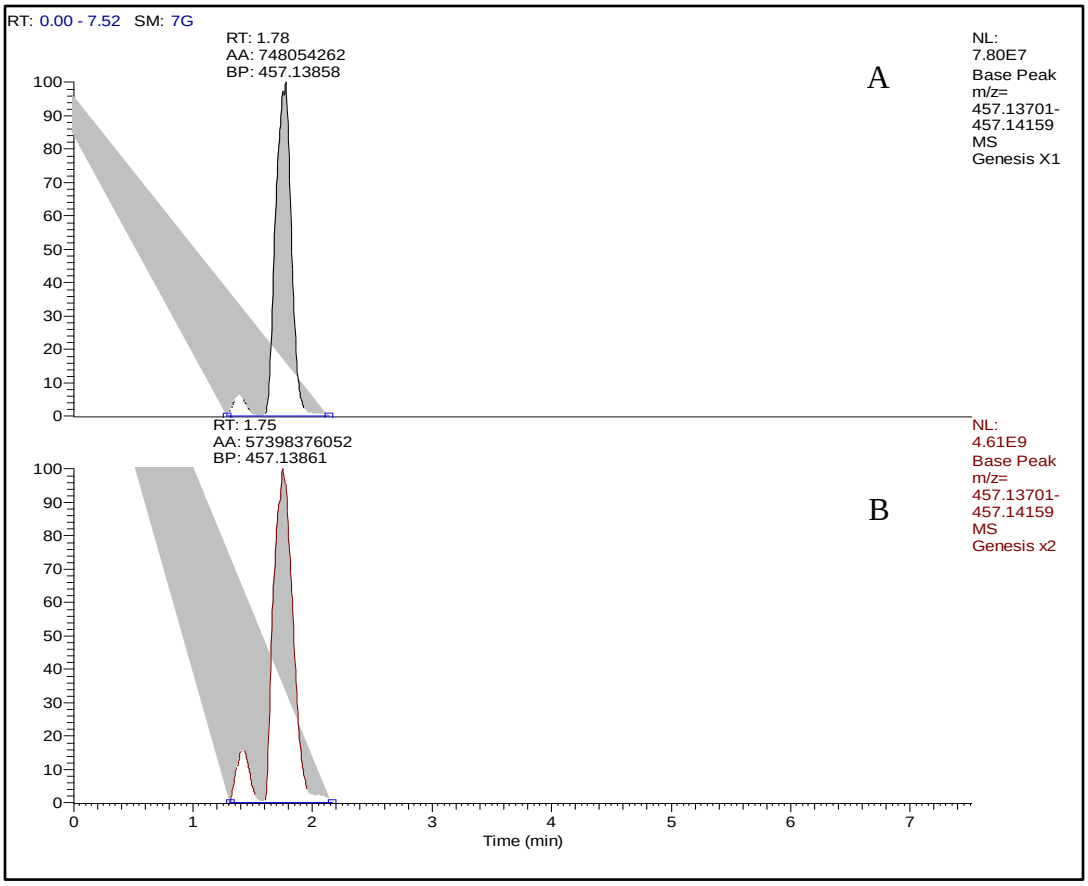

Figure 3. 18. **The UPLC Chromatograms of Two Residues from the Synthesis of NAPQI-Glutathione**. A. The target peak of NAPQI-GSH shown in the selected ion chromatograms for the residue from the methanol extraction of the crude synthesized NAPQI-GSH, B. the target peak of NAPQI-GSH shown as the selected mass for the residue from the evaporation of methanol from the crude synthesized NAPQI-GSH.

III. H. ii. Test of Isolated Standard NAPQI-GSH Complex from the Stock

The LC-MS chromatograms of the base peaks (without selection of a certain mass) implicated the most probable target peak for the NAPQI-GSH conjugate was more abundant in HPLC Fraction. C. (Fig. III. 19. C) The target peak of NAPQI-GSH was shown in the chromatogram at between 1.6 min and 2.2min, with a molecular mass of

about 457.14 amu. Fraction. C was thus stored and used as standard of NAPQI-GSH.

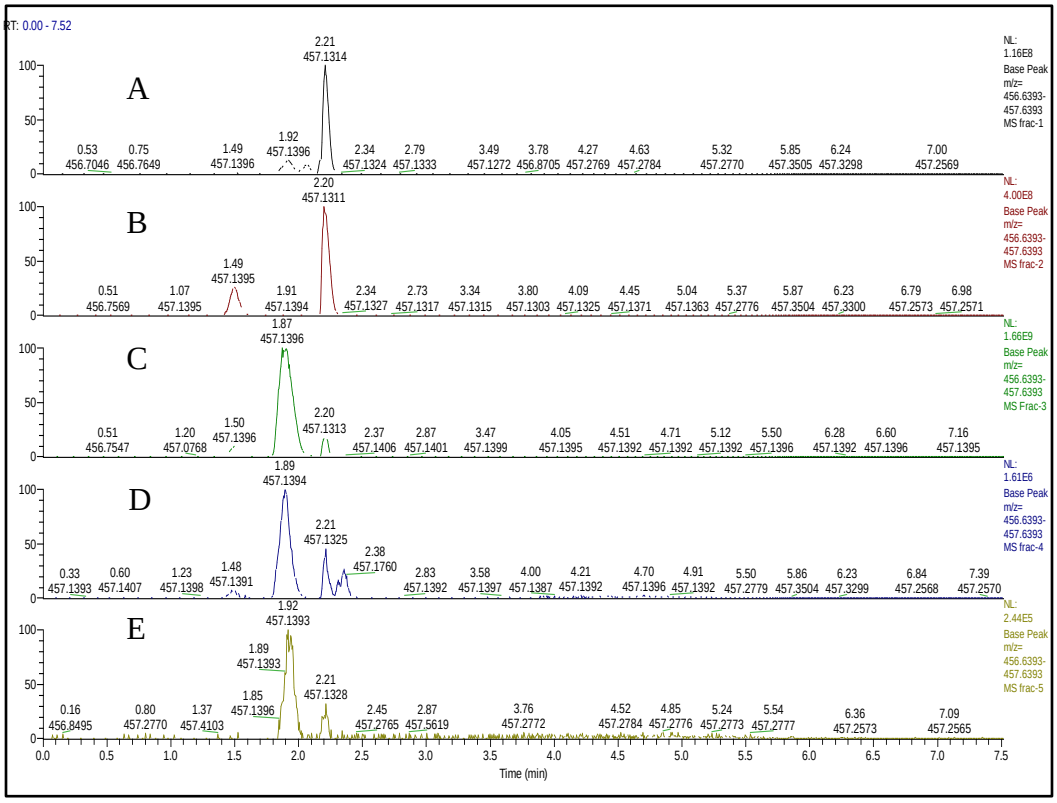

Figure 3. 19. LC-MS Chromatograms of Five Fractions of HPLC Separation Products of Stock Solution Containing NAPQI-GSH Conjugate. A. The fraction obtained from eluent at 2.5min; B. The fraction obtained from eluent at 3.0min; C. The fraction obtained from eluent from 3.5min to 4.0min; D. The fraction obtained from eluent from4.5min to 5.0min; E. The fraction obtained from eluent from 9.0 min to 12.0min.

III. H. iii. Detection of NAPQI-GSH in Enzyme Assay

The target peaks of the ionized NAPQI-GSH and its isomer showed a molecular mass of around 457.14 amu. For the samples of the positive trials to which NADPH was added, the peak for the protonated NAPQI-GSH and its isomer appeared at between 0.6

min and 0.7 min and at between 1.6 min and 2.2 min (Fig. III. 20. C, D). In contrast, for the chromatograms of the negative controls (Fig. III. 20. A,B), there were no peaks that appeared at these two times.

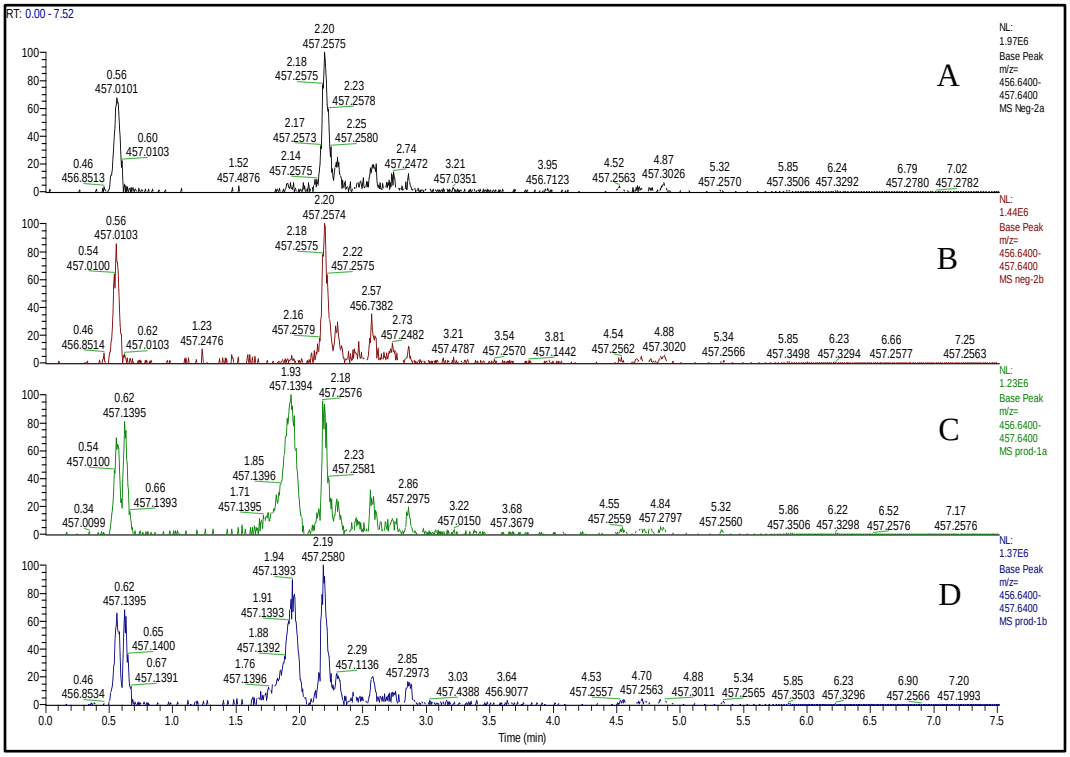

Figure 3. 20. LC-MS Chromatograms of Detection of NAPQI-GSH Formation. The formation of the complex was catalyzed by CYP2E1 and glutathione-S-Transferase from Human Liver Cytosol. A, B: the negative controls without adding NADPH; C, D: the positive trials with presence of NADPH. For the positive trials, there were two peaks of the target NAPQI-GSH which were eluted between 0.6 min and 0.7 min and between 1.6 min and 2.2 min.

III. H. iv. Inhibition of Purified Human Liver 2E1 by Ginger Hydrosol in Metabolism of Acetaminophen

The metabolism of acetaminophen by purified human liver 2E1 was shown to be inhibited by Brazilian ginger hydrosol when acetaminophen was added at the dose of 800μM. As shown in Figure 3. 21, the inhibition of human liver became stronger as the dose of Brazilian ginger hydrosol was increased. With the presence of ginger hydrosol at the concentration of 25%, a close to 50% inhibition in the enzyme activity in acetaminophen metabolism was observed. This concentration of Brazilian ginger hydrosol corresponds to a dose of citral of around 158 μM.

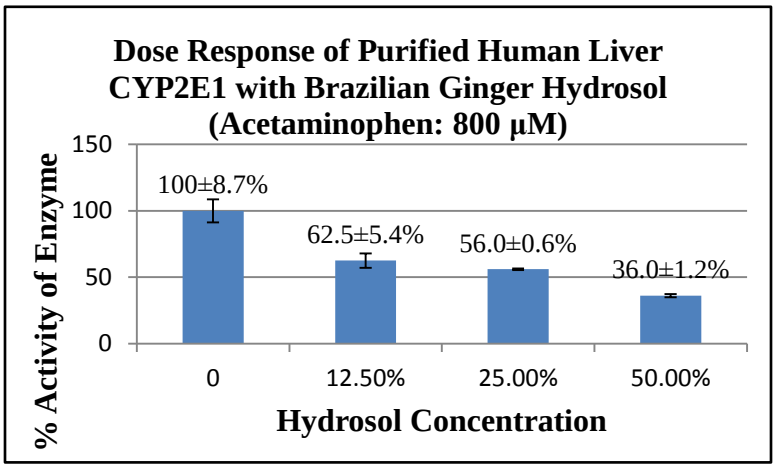

Figure 3. 21. Dose Responses of Purified Human Liver 2E1 to Brazilian Ginger Hydrosol in Acetaminophen Metabolism. The dose of acetaminophen as CYP2E1 substrate was 800 μM.

III. I. Michaelis-Menten Kinetics of Rabbit Liver CYP2E1 with Ginger Extracts and Citral

The Michaelsis-Menten kinetic analysis of rabbit liver was carried out with presence of ginger hydrosols, ginger essential oils and citral with three different isomer ratios. The purpose was to determine the types of inhibition (competitive, non-competitive or mixed) and the corresponding inhibitory constants. For the competitive inhibition, the inhibitory constant KI was calculated with the equation of $K_I=K_M*[I]/(K_M^{app}-K_M)$; for the non-competitive inhibition, the inhibitory constant KI' was calculated with the equation of $K_I'=[I]*V_{max}^{app}/(V_{max}-V_{max}^{app})$. For the rabbit liver CYP2E1, the ginger essential oils were determined to be competitive inhibitors. The inhibitory constant for Brazilian ginger essential oil was

$K_I'=[I]*V_{max}^{app}/(V_{max}-V_{max}^{app})=(19.2mg/L*68.4\%)/(100.0\%-68.4\%=41.6mg/L.$

The constant for the Costa Rican ginger essential oil was calculated as $K_I'=62.2mg/L$ (Figure 3. 22 and Table 3. 3).

In comparison, the ginger hydrosols were determined to have mixed inhibition to the rabbit liver CYP2E1 and they have both a competitive inhibitory constant and a non-competitive inhibitory constant. For example, the competitive inhibitory constant for the

Brazilian ginger hydrosol was calculated 6.3% of its original concentration, which corresponded to 78.7μM citral:

$K_I=K_M*[I]/(K_M^{app}-K_M)=(74.1\mu M*7.5\%)/(163.0\mu M-74.1\mu M)=6.3\%$.

Its non-competitive inhibitory constant was 11.7% of its original concentration, which corresponded to 112.9μM citral (Figure 3. 22 and Table 3. 3).

For rabbit liver CYP2E1 added with citral, it seemed that a shift in the neral: geranial proportion could cause a shift in the Michaelis-Menten kinetics of rabbit liver CYP2E1. At a relatively higher neral to geranial proportion like 1:1.27, citral exerted non-competitive inhibition on the rabbit liver CYP2E1 with K_I=166μM. When the ratio of geranial was increased to a adequately high value like neral: geranial=1:1.8, the inhibition of the rabbit liver CYP2E1 by citral turned out to be competitive with K_I=83μM. Noticeably, once the neral to geranial ratio was selected as a value in between, say, 1: 1.47, the inhibition of the rabbit liver CYP2E1 by citral became a mixed inhibition with the competitive constant K_I=140μM and the non-competitive K_I'=487μM (Figure 3. 23 and Table 3. 2). Therefore, it seemed that an increase of geranial proportion in citral from a higher neral to geranial ratio could shift the citral from a non-competitive inhibitor to a competitive inhibitor to the rabbit liver CYP2E1.

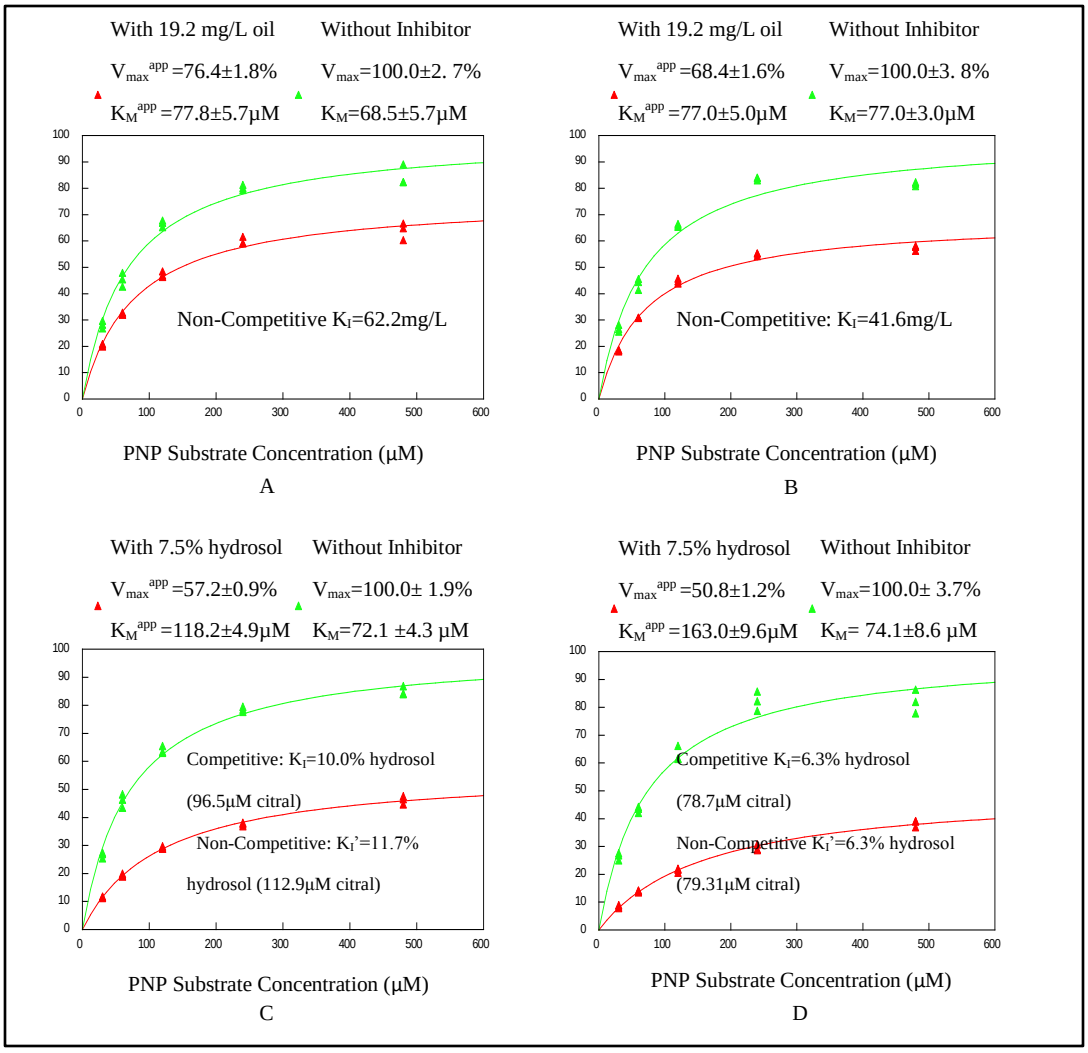

Figure 3. 22. Michaelis-Menten Curves of Rabbit Liver CYP2E1 with Ginger Extracts. A. Costa Rican ginger essential oil. B. Brazilian ginger essential oil. C. Costa Rican ginger hydrosol. D. Brazilian ginger hydrosol.

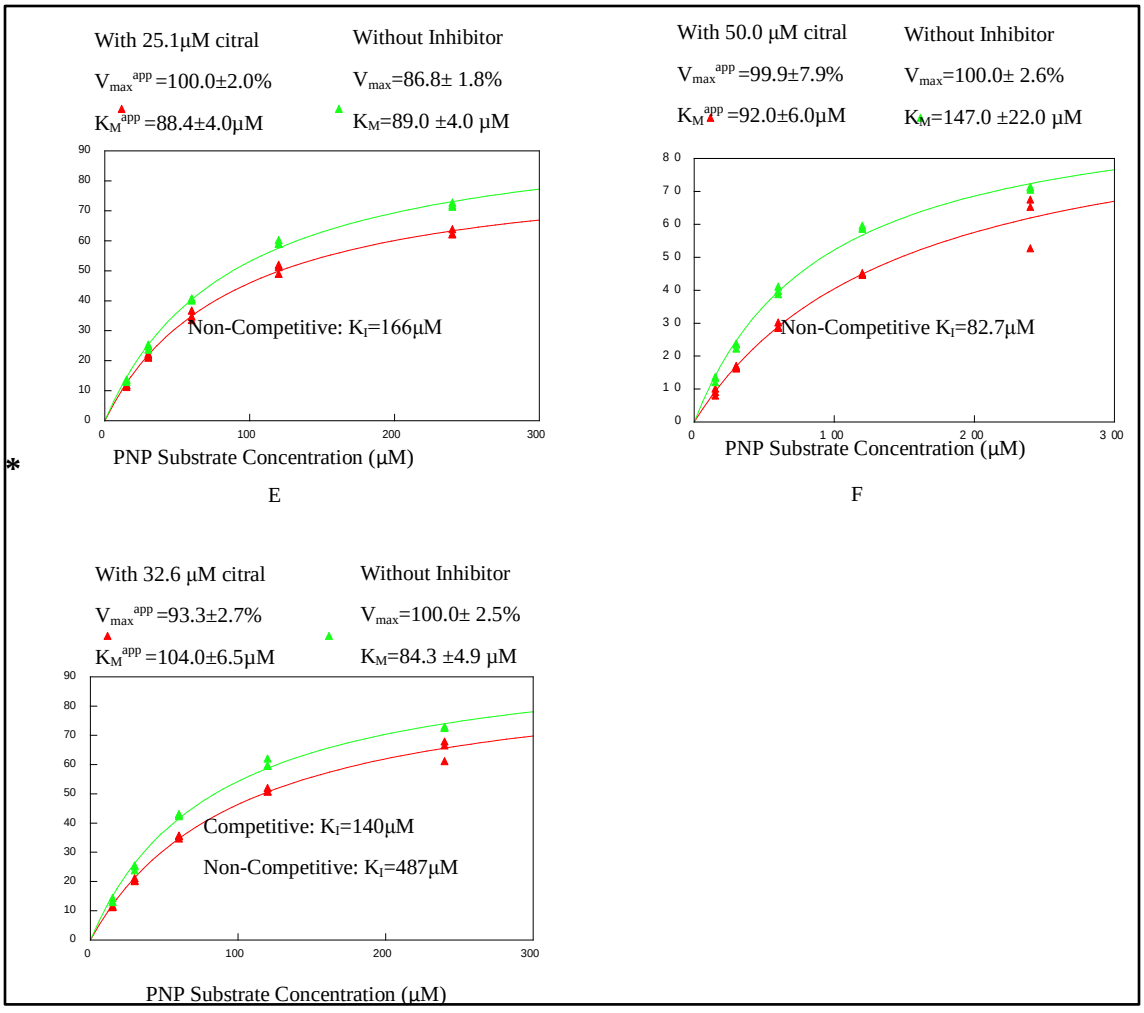

Figure 3. 23. **Michaelis-Menten Curves of Rabbit Liver CYP2E1 with Citral. A.** Citral (neral: geranial= 1: 1.27). B. Citral (neral: geranial= 1: 1.8). C. Citral (neral: geranial= 1: 1.47).

Table 3. 3. Michaelis-Menten Kinetics of Rabbit Liver CYP2E1 with Ginger Extracts and Citral Standards.

Inhibitor	Neral: Geranial	Inhibition Type & Ki
Costa Rican Ginger Essential Oil	1:1.8	Non-Competitive K_I=62.2mg/L
Brazilian Ginger Essential Oil	1:1.8	Non-Competitive K_I=41.6mg/L
Costa Rican Ginger Hydrosol	1:1.4	Mixed Inhibition; Non-Competitive K_I=10% hydrosol (96.5μM citral) Competitive K_I'=11.7% hydrosol (112.9μM citral)
Brazilian Ginger Hydrosol	1:1.4	Mixed Inhibition; Competitive K_I=6.3% hydrosol (78.7μM citral) Non-Competitive K_I'=6.3% hydrosol (79.31μM citral)
Citral	1:1.27	Non-Competitive K_I=166μM
Citral	1:1.8	Competitive K_I=83μM.
Citral	1:1.47	Mixed inhibition; Competitive K_I=140μM Non-Competitive K_I'=487μM

CHAPTER IV

CONCLUSION

Ginger extracts, no matter essential oil or hydrosol, were determined to be inhibitors of cytochrome 450 2E1. Ginger extracts made from steam distillation exerted significant inhibitions on CYP2E1 but poor inhibition on CYP2A6. Ciral was finally identified as the predominant human liver 2E1 inhibitor presenting in ginger hydrosol from the Michaelis-Menten kinetic analysis. Citral also demonstrated inhibition on metabolism of acetaminophen by human liver 2E1 into NAPQI. Therefore, citral might be used to physiologically provide with natural prevention of human liver toxicity via inhibition of liver CYP2E1 in generating NAPQI from metabolism of acetaminophen. However, to finally verify the possible clinical value of citral in this scenario, further evidence is still needed from in vitro cell assays and in vivo assays. The probable future test could be carried out by assay of 2E1 over-expressed HepG2 cells with presence of citral.

The Michaelsis-Menten kinetics of rabbit liver CYP2E1 was a branch research of this project. For the rabbit liver CYP2E1, ginger extracts and citral had shown more diverse inhibitory kinetics. Moreover, an increase of geranial proportion in citral from a

higher neral to geranial ratio could shift citral' inhibitory kinetics on the rabbit liver CYP2E1.

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